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A TEN YEAR OLD STRAIN OF FIBROBLASTS.

By ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 64 TO 66.

(Received for publication, January 26, 1922.)

A strain of fibroblasts, obtained from the heart of a chick embryo on January 17, 1912, has completed the 10th year of its life *in vitro*. On January 17, 1922, our incubators contained about 60 cultures which represented the 1860th generation of the connective tissue cells. The purpose of this paper is to describe the actual condition of the strain, to discuss its meaning, and to summarize the findings.

1. Present Condition of the Strain.—The growth of the tissue fragments is as rapid today as during the past years. Each fragment generally doubles its volume in 48 hours. The cultures (Fig. 1) have not modified their appearance.[1–4] They are composed of typical fibroblasts which grow in a dense tissue (Fig. 2) at the periphery of which individual cells may be observed and photographed (Figs. 3 and 4). Many cells divide mitotically (Figs. 5 to 8).

2. Unlimited Proliferation and Potential Immortality of Fibroblasts.—The fact that fibroblasts have been kept in active condition for 10 years has an important significance. It demonstrates that tissue cells living *in vitro* transform the foodstuffs of their medium into protoplasm, and that their mass increases effectively. Some experimenters believed that cells cultivated outside of the organism did not use the substances contained in the medium,[5] and that the mass of the tissue did not increase. This opinion applies to tissues cultivated in Locke

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

[2] Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273.

[3] Carrel, A., *J. Exp. Med.*, 1914, xx, 1.

[4] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277. Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421. Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

solution, or by the plasma method, but not to tissues cultivated in a medium containing embryonic tissue juices.[6] Observation of our strain of fibroblasts shows this beyond all doubt. In 10 years, more than 30,000 cultures have been derived from a fragment of heart less than 1 c.mm. in size. If it had been feasible to multiply the tissues to their greatest possible extent, today their mass would be very much larger than the sun. Thus it is evident that some of the food material contained in the medium has been transformed into protoplasm.

The existence of the 10 year old strain demonstrates also that the cells are potentially immortal. This hypothesis was proposed long ago by Carrel when he observed that after 28 months of life *in vitro* the rate of growth of the active tissue cells had not decreased.[3] It was evident that fibroblasts living under the conditions of his experiments were no longer subjected to the influence of time, as they are when living within the organism. They could be compared to unicellular organisms, such as *Paramecium*, which Woodruff has cultivated for over 14 years,[7] and could be considered as potentially immortal. This opinion was criticized by Harrison,[8] who considered it unjustified until the cells should have lived in cultures for a period exceeding the duration of life of the organism from which they were taken. Pearl[9] believes that the present age of the strain of fibroblasts fully disposes of this criticism, as the limit of the life of chickens is often 10 years. It may be considered as certain, therefore, that fibroblasts will proliferate indefinitely, as do colonies of Infusoria.

3. Use of the Strain of Fibroblasts in Physiological Studies.—The purpose of the experiments was not only to ascertain whether fibroblasts could proliferate indefinitely, but also to study certain biological problems. Pure cultures of cells[10] are as necessary in physiology as pure cultures of microorganisms in bacteriology. The strain, being composed only of typical fibroblasts, grows with great regularity, and was found to respond readily to changes in the composition of the culture medium by a modification of its rate of proliferation. The

[6] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.
[7] Woodruff, L. L., cited by Pearl, R., *Scient. Monthly*, 1921, xii, 202.
[8] Harrison, R. G., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 71.
[9] Pearl, R., *Scient. Monthly*, 1921, xii, 332, 333.
[10] Carrel, A., *J. Exp. Med.*, 1912, xvi, 165.

original technique[1,11] was progressively perfected. Studies of the optimum medium from the point of view of osmotic tension[12] and H ion concentration[13] were made. Substitution of a fibrinogen suspension for plasma was rendered possible.[14] More accurate methods of preparing and measuring the cultures were developed,[15] by which differences of less than 10 per cent in the rate of growth could be ascertained.

The strain, then, could be used as a reagent for the detection of substances contained in the humors which have the power of activating or decreasing the rate of cell proliferation. The variations of the rate of growth demonstrated the presence in embryonic juice of a factor which increases the velocity of cell multiplication to a high degree.[6,16] It showed also that the state of the fibroblasts at a given time was function of two independent variables, their dynamic condition at the preceding instant, and the condition of the medium.[17] It became evident that the activity of the fibroblasts does not depend on the amount of potential energy they contained at the beginning of their life but upon certain substances present in the medium. The cells remained indefinitely young or grew old[17] according to the food material they were given and the extent of the elimination of their catabolic substances.

In the same manner, it was found that adult serum produces on fibroblasts an effect opposite to that of embryonic tissue juice. This inhibiting power of a serum increased very much with the age of the animal from which it was obtained.[18] As there was a definite relation between the age of the animal and the rate of cell proliferation in its serum, the growth index of the serum indicated the variations in the age.[19]

[11] Carrel, A., *J. Am. Med. Assn.*, 1911, lvii, 1611.
[12] Ebeling, A. H., *J. Exp. Med.*, 1914, xx, 130.
[13] Fischer, A., *J. Exp. Med.*, 1921, xxxiv, 447.
[14] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.
[15] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.
[16] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.
[17] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.
[18] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxv, 17.
[19] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

The strain was used also in the study of the response of tissue cells to an antigen. It is known that an antigen added to a culture of bone marrow determines the formation of an antibody.[20] When the strain was sensitized by a foreign protein, its response to a second injection[21] showed that it could be used in the investigation of certain phenomena of immunity. As it has become possible lately to obtain strains of lymphocytes[22] and of epithelial cells[23] living *in vitro* by practically the same procedure that is used for fibroblasts, the scope of these studies will be increased.

EXPLANATION OF PLATES.

PLATE 64.

FIG. 1. Culture 24871–1. Passage 1858. 10 years, lacking 4 days, of life *in vitro*. Portion of the central and peripheral zone. Among the peripheral cells, a number of mitoses can be distinguished. Stained with methylene blue after 48 hours growth. × 100.

PLATE 65.

FIG. 2. Culture 24870–1. Passage 1858. 10 years, lacking 4 days, of life *in vitro*. Very active culture. Peripheral area in which nine mitotic figures can be observed. Stained with methylene blue after 48 hours growth. × 300.

PLATE 66.

FIG. 3. Culture 24871–1. Passage 1858. Typical fibroblast in the peripheral zone of the culture shown in Fig. 1. × 1,000.

FIG. 4.[24] Culture 422. Passage 1710. 9 years, 3 months, lacking 4 days, of life *in vitro*. Typical fibroblast. Stained with gold chloride after 48 hours growth. × 1,100.

FIGS. 5 and 6. Culture 24041–2. Passage 1844. A cell undergoing mitotic division after 10 years, lacking 1 month, of life *in vitro*. Stained with methylene blue after 48 hours growth. × 1,000.

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[20] Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

[21] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 661.

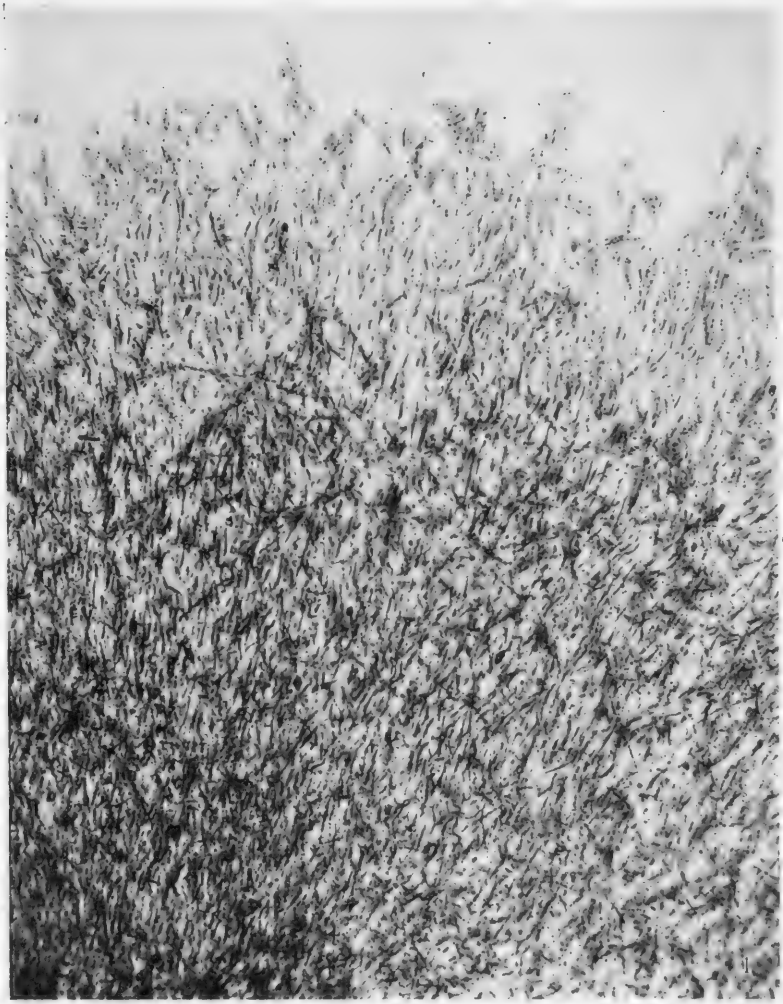
[22] Carrel, A., and Ebeling, A. H., unpublished experiments.

[23] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

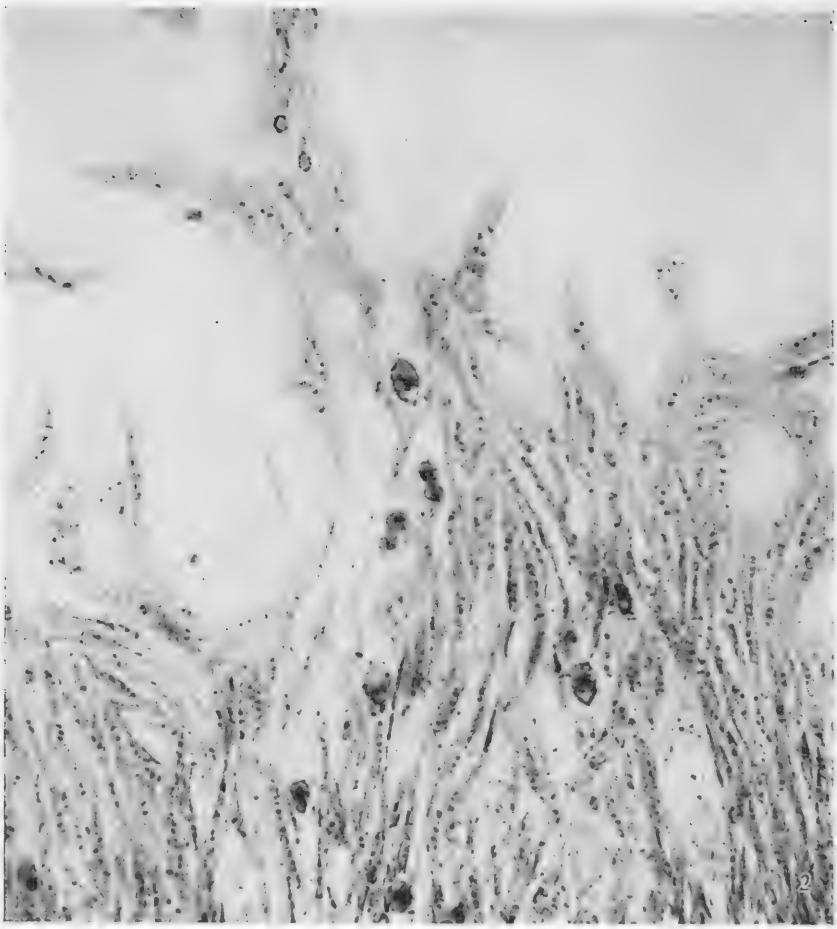
[24] I am indebted to Dr. Albert Fischer for permission to show a picture of this particular preparation.

FIG. 7. Culture 964 A. Passage 1857. 10 years, lacking 2 days, of life *in vitro*. Fibroblast a short time after division. Stained with methylene blue after 48 hours growth. × 1,000.

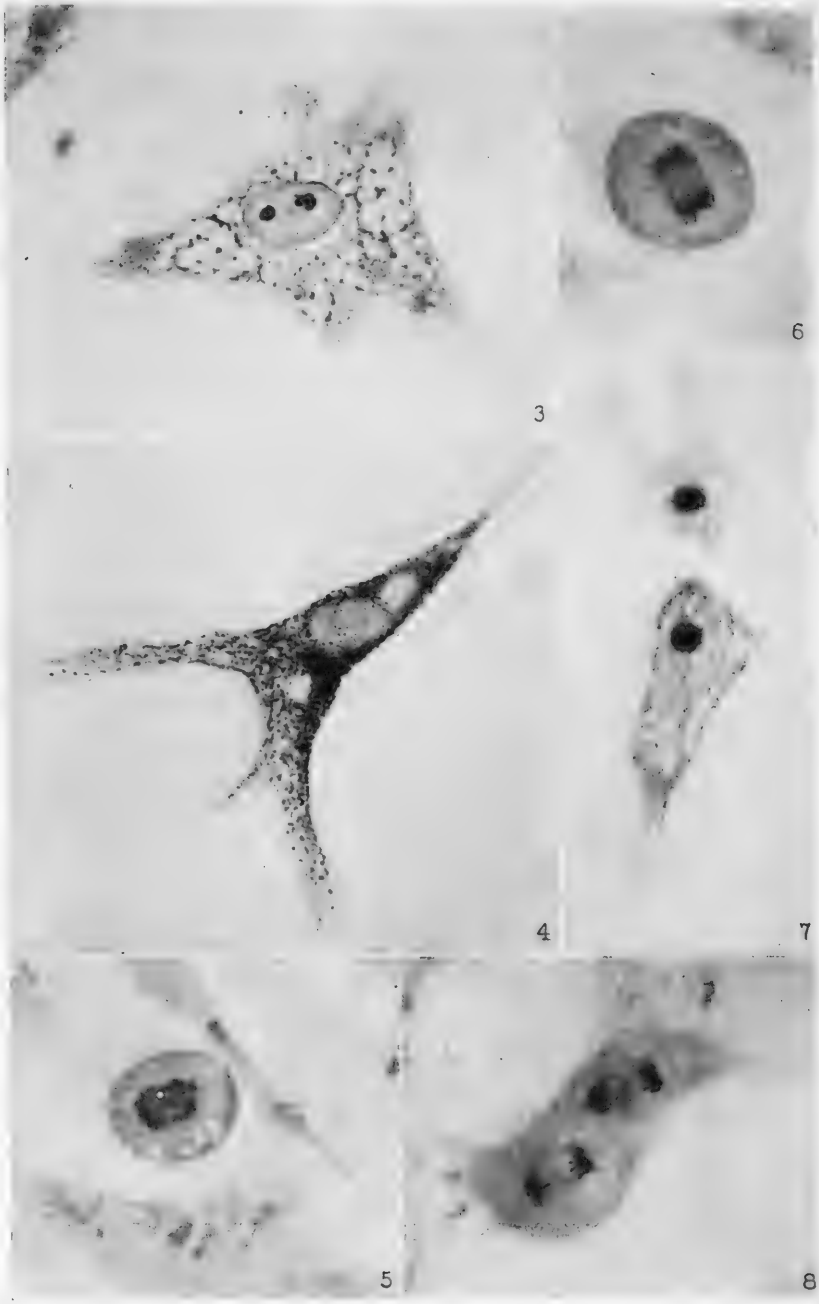
FIG. 8. Culture 964 A. Passage 1857. Double mitoses in an actively growing culture, after 10 years, lacking 2 days, of life *in vitro*. Stained with methylene blue after 48 hours growth. × 1,000.



(Ebeling: Ten year old strain of fibroblasts.)



(Ebeling: Ten year old strain of fibroblasts.)



(Ebeling: Ten year old strain of fibroblasts.)

STUDY OF THE ACTION OF FOUR AROMATIC CINCHONA DERIVATIVES ON PNEUMOCOCCUS. A COMPARISON WITH OPTOCHIN.

By LLOYD D. FELTON, M.D., and KATHARINE M. DOUGHERTY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 12, 1922.)

Since Morgenroth[1] introduced ethylhydrocupreine (optochin) as a chemotherapeutic agent against the pneumococcus, a number of related compounds has been investigated in the effort to find one more distinctly monotropic. However, up to the present, optochin holds a unique position in that it alone has been proven to influence beneficially an experimental pneumococcus infection in mice, in which animal the bacteriotropism is greater than the coincident organotropism. But this difference in the balance between organotropic and bacteriotropic action of optochin is insufficient to establish a definite therapeutic effect once the pneumococcus infection has become systemic in nature. The use of optochin against pneumococcus infection in man has had a rather comprehensive trial. In pneumonia, the results of investigations of Moore and Chesney,[2] Manliu,[3] Wright,[4] Parkinson,[5] and others have shown its use in this connection to be of little value. This rather discouraging outcome has shown itself to be true, despite the increase in the bactericidal power of the patient's blood (Moore[2] and Wright[4]) and the negligible change in opsonic index (Wright[4]) following its use. Bier[6] has claimed therapeutic effect in treatment of superficial wounds. Uncertain results in pneumococcus meningitis of man were reported by Wolff and Lehmann,[7] Lippman,[8] and Rosenow,[9] while Cordua[10] has found a favorable action in cases of

[1] Morgenroth, J., and Levy, R., *Berl. klin. Woch.*, 1911, xlviii, 1560, 1979. Morgenroth, J., *Naturwissenschaften*, 1913, i, 609. Morgenroth, J., and Bieling, R., *Berl. klin. Woch.*, 1917, liv, 723.

[2] Moore, H. F., and Chesney, A. M., *Arch. Int. Med.*, 1917, xix, 611; 1918, xxi, 659.

[3] Manliu, J., *Berl. klin. Woch.*, 1916, liii, 58.

[4] Wright, A. E., *Lancet*, 1912, ii, 1633, 1701.

[5] Parkinson, C., *Z. Chemotherap., Orig.*, 1913, ii, 1.

[6] Bier, A., *Berl. klin. Woch.*, 1917, liv, 717.

[7] Wolff, S., and Lehmann, W., *Jahrb. Kinderheilk.*, 1914, lxxx, 188.

[8] Lippman, *Berl. klin. Woch.*, 1917, liv, 781.

[9] Rosenow, G., *Deutsch. med. Woch.*, 1920, xlvi, 9.

[10] Cordua, R., *Berl. klin. Woch.*, 1921, xlviii, 1323.

meningococcus meningitis. However, Kolmer and Idzumi[11] were unable to produce a favorable influence on an experimental pneumococcus infection of the meninges of dogs and rabbits.

The realization of the limitations of this compound led us to investigate the cinchona derivatives synthesized and reported by Jacobs and Heidelberger.[12] It seemed to us that regardless of whether or not a substance was found that possessed greater activity than optochin, a biological study of these numerous compounds—so closely related to optochin—might be an aid in further chemical and biological investigations.

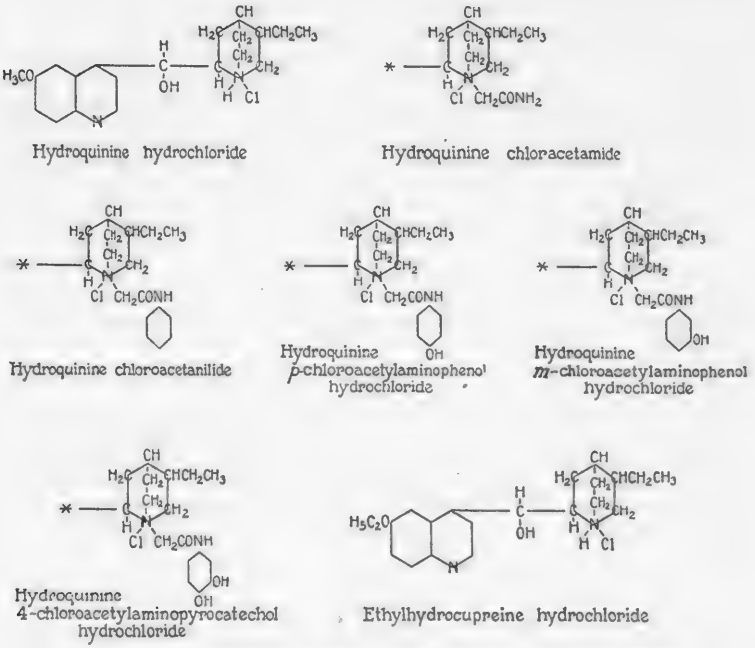
It was found at the outset that certain of these modified cinchona compounds possess a remarkably high bactericidal activity for pneumococci *in vivo*. In this paper we wish to present a study of four active chemicals in comparison with optochin. These four were chosen, as is shown below, for the reason that they represent a uniform series of hydroquinine derivatives, thus affording opportunity for a comparison between the chemical constitution and biological activity of closely related compounds.

To show clearly the relationship between these compounds and the exact chemical differences, structural formulas are given in Text-fig. 1. The first change in hydroquinine is the addition of chloracetamide on the quiunclicine ring, making hydroquinine chloracetamide. In turn, one of the amide hydrogens of the acetamide group is substituted by a benzene nucleus forming hydroquinine chloroacetanilide, the first important member of this series. The remaining drugs are hydroxy substitution products of the latter, in which the OH group is substituted in the para or the meta, or in both para and meta positions in the benzene nucleus, yielding respectively hydroquinine *p*-chloroacetylaminophenol hydrochloride, hydroquinine *m*-chloroacetylaminophenol hydrochloride, and hydroquinine 4-chloroacetylaminopyrocatechol hydrochloride. To avoid repetition of these names in this paper, the laboratory number will be used, as follows: the chloroacetylanilide, C 29; the *p*-chloroacetylaminophenol, C 36; the *m*-chloroacetylaminophenol, C 40; and the 4-chloroacetylaminopyro-

[11] Kolmer, J. A., and Idzumi, G., *J. Infect. Dis.*, 1920, xxvi, 355.

[12] Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1919, xli, 2090.

catechol, C 110. As can be seen from the chart, these substances are derived from the hydroquinine and not from the ethylhydrocupreine nucleus, the only difference in the two compounds being that one is a methoxy and the other an ethoxy substitution on the quiniline nucleus. There is in these chemicals a combination of two bactericidal compounds—the quinines and benzenes. Since Lister introduced



TEXT-FIG. 1. Structural formulas of the cinchona alkaloids studied.

phenol into antiseptic surgery practically all the hydroxybenzene compounds have been employed for the same or similar purposes, but abandoned for one reason or another as impracticable. These new organic chemicals can therefore be thought of either as additive compounds of the benzene derivatives to the hydroquinine nucleus or the hydroquinine to the benzene nucleus by means of a common linkage CH_2ONH.

General Procedure.

Since we undertook to make a comparative study of the different chemicals, work was planned so that each experiment, in so far as was practical, embraced all the drugs under the same conditions. Young mice of a healthy stock, weighing from 15 to 18 gm., were employed throughout. Animals of this weight were used because it was early found that large, and especially old, female mice reacted irregularly. Doses of the chemical were approximated to 18 gm. mice. Each mouse was autopsied at death and one loop of heart's blood smeared on a blood agar plate, presence or absence of growth being noted at the end of 24 hours. Animals that survived were kept for 30 days and then killed. Cultures were made from a few animals living the 30 day period, but inasmuch as no pneumococci could be found, this procedure was abandoned. Unless otherwise stated in the tables pneumococci were present at death in the blood stream of all mice recorded. Since we found considerable variation in numbers of organisms in both the treated animals and the controls, little stress was laid on this factor. For each experiment, controls were run in duplicate with what had been found from previous experience to be 1, 10, and 20 M.L.D. This minimum lethal dose was calculated each time for the given experiment after the death of the controls, and was taken to be the smallest number of organisms, or the highest dilution of the culture (made in physiological salt solution), causing the death of duplicate mice in 48 hours.

The chemicals were dissolved in boiling sterile distilled water. The pneumococcus used was a Type I (Neufeld).[13] Its virulence was such that generally two to four organisms (fluctuations occurring from time to time from two to twenty organisms) of a 6 hour culture, as determined by plating, injected into the peritoneal cavity of a mouse, resulted in the death of the animal within 48 hours. The intravenous virulence of this organism was rather low, requiring approximately 200,000 diplococci to cause death, or 10,000 times as many as by the intraperitoneal route.

Solubilities.

In Table I are given the solubilities of the chemicals in water, physiological salt solution, and serum. They have a practical bearing as can be seen below, in view of the bactericidal results obtained *in vivo* and *in vitro*. In making the determinations with water and physiological salt solution, each respectively was added slowly in measured amounts on 100 mg. of substance until solution was obtained. The solution in serum represents the concentration of the drug that does not cause precipitation in the serum, and can hardly be termed solubility. Both the aromatic compounds and optochin

[13] The strain of pneumococcus was kindly furnished by Dr. O. T. Avery.

are protein precipitants. C 29 is the least and optochin the most soluble, while C 36, the para-hydroxybenzene derivative, is more soluble than C 40, with the OH group in the meta position. C 110, having the OH group both in the para and meta positions, holds an intermediate position between these two compounds.

TABLE I.

Solubilities.

Chemical.	Percentage of chemical in solvent.		
	Water.	Physiological salt solution.	Horse serum.
C 29	0.3	0.12	0.06
C 36	10.0	10.0	0.12
C 40	2.0	2.0	0.05
C 110	4.0	4.0	0.25
Optochin.	10.0	10.0	0.4

Bactericidal Action in Vitro.

Bactericidal action has been measured in two ways, (*a*) by a constant dose of organisms inoculated into varying concentrations of the chemical and (*b*) by a constant concentration of drug to which were added varying numbers of organisms. Since Chesney[14] has shown

TABLE II.

*Bactericidal Action in Whole Blood in Vitro.**

Chemical	C 29	C 36	C 40	C 110	Optochin.
Dilution sterile in 2 hrs.	1:1,500	1:750	1:1,000	1:500	1:8,000

* Neufeld pneumococcus.

that a young culture is more resistant to bactericidal agents than an old one, a 6 hour culture of the Neufeld pneumococcus was employed in these tests, in order that conditions might resemble as nearly as practicable those *in vivo*. In the first method whole defibrinated blood was used as medium.

[14] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

To 2 cc. of the blood was added the chemical in water solution with concentrations adjusted to give a constant volume in making the following series of dilutions: 1:500, 1:750, 1:1,000, 1:1,500, 1:2,000, 1:4,000, 1:8,000, and 1:12,000. This series was inoculated with 0.2 cc. of a broth culture per tube and incubated for 2 hours in a water bath at 37°C. One loopful from each tube was then distributed over one-fourth of a blood agar plate. These plates were incubated for 96 hours and read. From Table II it can be seen that under these conditions the aromatic derivatives are less active than optochin, the latter being about eight times as potent as C 29, the best of the others.

In the second method, bactericidal activity was determined by exposing the organisms in physiological salt solution, 50 per cent serum, and 50 per cent defibrinated rabbit blood, to the action of the chemicals for 5 minutes.

The different dilutions of pneumococci were made in 0.4 cc. of physiological salt solution, undiluted serum, and whole blood, respectively, by adding 0.1 cc. of the appropriate dilution of the culture to make the final concentration the one desired. The chemicals were dissolved in water, so that 0.5 cc. contained 1 mg. of substance. In the final test (Table III) there was a volume of 1 cc. with 1 mg. of chemical and a series of dilutions of a 6 hour culture of 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000. The controls in each case were made at the end of the experiment, 0.5 cc. of physiological salt solution being added to make the volume up to 1 cc. To each tube containing 0.5 cc. of culture was added 0.5 cc. of the chemical. The mixture was put in the water bath at 37°C. for 5 minutes, and then 0.05 cc. was pipetted into 100 cc. of meat infusion broth (pH 7.4), thus bringing the chemical dilution above its bactericidal potential (1:2,000,000). The flasks were under observation for 4 days and results recorded each 24 hour period except for the test on blood, the first observation in this case being made 12 hours after inoculation. Controls all grew out in 24 hours, while in this length of time no visible growth occurred in the flasks containing organisms subjected to the chemicals. The order of the rapidity of bactericidal action in decreasing ratio is as follows: C 29, C 40, C 36, optochin, and C 110. Neither serum nor blood diminished the activity of C 29 or optochin. The unchanged benzene nucleus derivative possesses the most rapid action; the one with the OH group in the meta position is more active than the one with the OH in the para position; while the compound with the OH group in both meta and para positions is still less active. Optochin in this experiment proves to have a slow rate of activity, as had been found by Solis-Cohen, Kolmer, and Heist.[15]

In addition, the dilution of the drugs that inhibited bacterial growth, with resultant death, was determined on meat infusion broth (pH 7.8).

[15] Solis-Cohen, S., Kolmer, J. A., and Heist, G. D., *J. Infect. Dis.*, 1917, xx, 313.

TABLE III.

Bactericidal Action with a 5 Minute Contact Period. 1 Mg. of Chemical Used in Physiological Salt Solution, Serum, and Whole Blood.

Chemical.	Dilution of pneumococcus (Neufeld).	Physiological salt solution.						50 per cent horse serum.						50 per cent defibrinated rabbit blood.					
		Chemical.				Control.		Chemical.				Control.		Chemical.				Control.	
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	12 hrs.	24 hrs.
C 29	1:10	0	+			+		0	+			+							
	1:100	0	+			+		0	+			+		0	0	+		+	
	1:1,000	0	0	0	0	+		0	+			+		0	0	0	0	+	
	1:10,000	0	0	0	0	+		0	0	0	0	+		0	0	0	0	+	
	1:100,000	0	0	0	0	±	+	0	0	0	0	±	+	0	0	0	0	±	+
C 36	1:10	0	+			+		0	+			+							
	1:100	0	+			+		0	+			+		+				+	
	1:1,000	0	+			+		0	+			+		+				+	
	1:10,000	0	0	0	0	+		0	+	+	+	+		0	+			+	
	1:100,000	0	0	0	0	±	+	0	0	0	0	±	+	0	+			±	+
C 40	1:10	0	+			+		0	+			+							
	1:100	0	+			+		0	+			+		+				+	
	1:1,000	0	0	0	0	+		0	+			+		+				+	
	1:10,000	0	0	0	0	+		0	0	0	0	+		0	+			+	
	1:100,000	0	0	0	0	±	+	0	0	0	0	±	+	0	+			±	+
C 110	1:10	0	+			+		0	+			+							
	1:100	0	+			+		0	+			+		+				+	
	1:1,000	0	+			+		0	+			+		+				+	
	1:10,000	0	0	+		+		0	0	+		+		+				+	
	1:100,000	0	0	0	0	±	+	0	0	0	0	±	+	0		+		±	+
Optochin.	1:10	0	+			+		0	+			+							
	1:100	0	+			+		0	+			+		+					
	1:1,000	0	+			+		0	+			+		0	+			+	
	1:10,000	0	0	+		+		0	+			+		0	0	0	+	+	
	1:100,000	0	0	0	0	+	+	0	0	0	0	±	+	0	0	0	+	±	+

0.1 cc. of a 6 hour culture was pipetted into each tube (10 cc. volume) of broth containing the series of dilutions represented in Table IV. The inoculated broth was incubated for 4 days, and then 1 cc. from the last tube showing no visible growth was plated for confirmation. Hydroquinine (C 9) and hydroquinine chloracetamide (C 20) were added to the experiment for comparison.

It is rather interesting to note that the substitution of the chloracetamide on the hydroquinine nucleus decreases its bactericidal activity. With the exception of optochin, the same relationship persists between this group of chemicals as in the preceding experiment. C 29 is as active as optochin under these conditions.

TABLE IV.

Bactericidal Action in the Presence of Broth (pH 7.8).

Chemical.	Dilution in broth.														
	1:2,500	1:5,000	1:10,000	1:15,000	1:20,000	1:30,000	1:40,000	1:60,000	1:80,000	1:120,000	1:160,000	1:240,000	1:320,000	1:480,000	1:640,000
C 9								+	++	++	++	++	++	++	++
C 20		++	++	++	++	++	++	++	++	++	++	++	++	++	++
C 29											+	++	++	++	++
C 36								++	++	++	++	++	++	++	++
C 40										++	++	++	++	++	++
C 110				+	++	++	++	++	++	++	++	++	++	++	++
Optochin.											++	++	++	++	++

Toxicity.

Instead of the toxic dose the largest tolerant dose is given, as determined by averaging several titrations (Table V). The injections *per os* were made by means of a small silver stomach tube attached to a syringe by flexible rubber tubing, the volume injected always being 0.5 cc. From the standpoint of the largest tolerant dose, the same relative positions of the aromatic compounds exist organotropically as were shown to exist parasitotropically; that is, with these four aromatic compounds toxicity runs parallel with bactericidal power—the most toxic, C 29, is the most bactericidal. If rapidity of bactericidal action is used as the criterion, this is also true of optochin. The intravenous toxicity is perhaps largely a physical phenomenon due to protein precipitation.

In the balance between the organotropism and parasitotropism lies the hope of chemotherapy. It is perhaps unreasonable to suppose that a drug will ever be discovered that is strictly monotropic in its action. Manifestly, all the drugs used in clinical medicine show distinctly a therapeutic dose beyond which they set up complex phenomena causing aggravation rather than amelioration. Just as a drug is active often because of its affinity for a certain monocellular tissue in the animal body, so must we look for a drug that has a greater toxicity for monocellular organisms—bacteria—than for any cells vital to the defensive mechanism of the animal. It is perhaps impossible, at this stage of our knowledge, to make this comparison. However, if the bactericidal action of the largest tolerant dose of these

TABLE V.

Toxicity.

Chemical.	Largest tolerant dose.			
	Intravenous.	Intraperitoneal.	Subcutaneous.	*Per os.*
	mg.	*mg.*	*mg.*	*mg.*
C 29	0.2	0.5	2.0	5.0
C 36	0.5	2.5	5.0	10.0
C 40	0.2	1.5	3.0	8.0
C 110	0.5	4.0	6.0	12.0
Optochin.	0.6	3.0	4.0	10.0

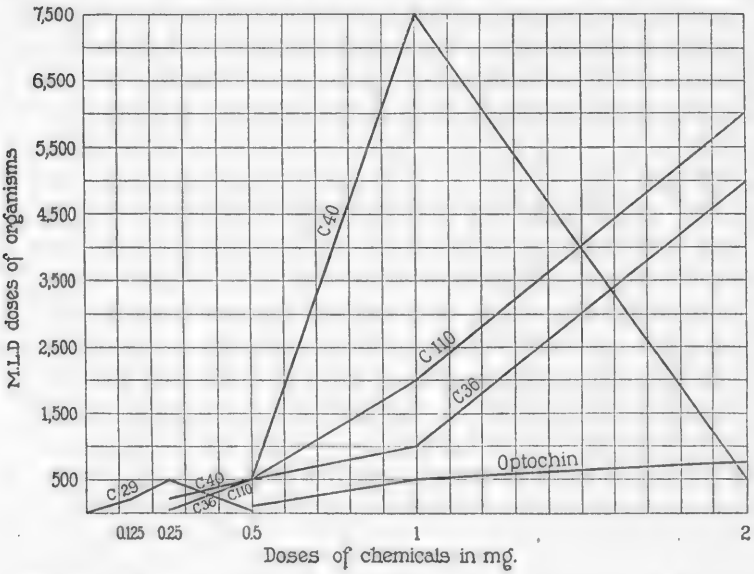
chemicals (Table V) for the experimental animal—the mouse—is compared with the number of virulent pneumococci that are killed *in vitro* (Table III) in as short a time as 5 minutes, the first step of this comparison is taken; that is, a non-toxic dose of the drug (for mice) is capable of killing *in vitro* many multiple lethal doses of virulent organisms. The following experiments were designed to show whether these relationships exist *in vivo*.

We wish to present the results of these *in vivo* experiments from a twofold standpoint. First, will the drugs kill multiple lethal doses of virulent pneumococci when chemical and organism are injected simultaneously into a walled off body cavity, such as the peritoneal? Second, will the drugs diffuse, and influence the experimental infection when injected by routes other than the one by which the animal was

infected? Briefly, is there either a local or a systemic bactericidal action *in vivo*, or both?

Simultaneous Injection of Multiple Lethal Doses of Pneumococci with Varying Amounts of Drugs.

The simplest possible procedure, perhaps, by which to estimate *in vivo* bactericidal activity resulting in an animal following the injection of a given drug is a simultaneous injection of organism and



TEXT-FIG. 2. Representation of the number of minimum lethal doses of organisms influenced by different doses of drugs—a therapeutic zone phenomenon.

chemical into the peritoneal cavity. By using as an indicator a microorganism of known virulence, it is possible to obtain within limits of biological accuracy an estimation of the number of organisms killed. The curves depicted in Text-fig. 2 are only approximate, owing to the wide range in dosage of organisms. These curves represent a titration of the number of minimum lethal doses of pneumococci killed by each of four doses of drugs, decreasing by halves from the

largest non-lethal dose. The number of minimum lethal doses of organisms, namely 1, 10, 100, and 1,000, in the preliminary titrations, was the same for all the doses of each chemical. Subsequently dilutions representing minimum lethal doses intermediate between the above doses were injected to furnish data given in Text-fig. 2. All titrations were made in duplicate.

The work with the aromatic compounds gave consistent results throughout. Optochin, on the other hand, varies in its effectiveness, especially in the 2 mg. dose. With this dose, the 740 M.L.D. from which the mice were protected is the average of five titrations: 400, 100, 200, and 1,000 to 2,000 M.L.D. The last two figures were obtained when the pneumococcus was at its greatest virulence, two to four organisms causing the death of the control in 48 hours. At the time of the other three experiments, 20 to 40 organisms were necessary to cause death of the controls in this interval. The fact that approximately ten times as many organisms were present in the unit injected at one time as at the other possibly accounts for the variable results. As the aromatic compounds are more rapid in their action, this may account for the fact that the results are more constant than with the more slowly acting optochin.

Hydroquinine has been shown by Morgenroth, Kolmer,[15] and Tugendreich and Russo[16] to have slight but distinct therapeutic value, though it is not so active as ethylhydrocupreine. By using the same technique as employed with these aromatic derivatives, it was found that hydroquinine protected against 100 M.L.D. at a time when this was represented by approximately 200 pneumococci. Hydroquinine chloracetamide, in the same experiment, protected against only 10 M.L.D. The results with these two chemicals show a parallelism between *in vitro* and *in vivo* bactericidal action (Table IV).

The very striking zonal reaction noted with C 29 and C 40 shows that there is an optimum concentration of the chemicals, below their toxic dose, at which virulent pneumococci are killed *in vivo*. If a therapeutic amount larger or smaller than this is injected, the mice die of septicemia. Manifestly, whatever the mode of action in this case may be, the normal defensive mechanism of the animal

[16] Tugendreich, J., and Russo, C., *Z. Immunitätsforsch., Orig.*, 1913, xix, 156.

is weakened, the relation between mono- and polytropism being reversed. This same phenomenon occurs with the other chemicals, as will be shown later in this paper.

Comparison of Single and Repeated Injections of the Same Amount of Drug.

To make a still more vigorous test on this local effect, experiments were carried out on single and repeated injections of drugs, 1 and 2 hours after the animal was experimentally infected.

It has been shown by many workers that it is more difficult to cure an infection if time elapses between inoculation of the animal and application of remedial measures. That this is true with these chemicals is shown in Table VI. The experiment thus represented was carried out to show the relative effectiveness between single doses, and the same amount divided into fifths. These two methods of treatment were applied to mice 1 hour after injection of organisms, those receiving a single dose of drug being injected with 10 M.L.D., while those having the dose divided were injected with 10, 20, and 50 M.L.D.

The single dose of the drug proved ineffective, except with optochin, in which case one mouse with 10 M.L.D. survived. The repeated divided dose presented a different picture, as many as 50 M.L.D. of the organisms being killed with the aromatic compounds. Optochin in these small doses was unable to cure an animal infected with more than 10 M.L.D. The drop in the effectiveness of the chemicals when injected 1 hour after the organism is remarkable. The simultaneous procedure with C 40, for instance, proved to be lethal for 8,000 M.L.D. of organisms; injections deferred for 1 hour had no influence on 10 M.L.D. The power of the drug was thus decreased approximately 8,000 times.

Inasmuch as optochin, under these conditions, did not hold up as well as the other derivatives, the possibility was suggested that dosage was at fault. To establish this point and to determine the optimum concentration for five hourly injections, the experiment represented in Table VII was carried out.

2 hours after the injection of the pneumococci, a series of five injections of each doses of the chemicals, at intervals of 1 hour, was begun. The repeated 1 mg. doses of C 36 and C 40 were toxic for the mice. However, at death the C 36 animals had pneumococci in their blood streams, while with C 40, the animal with

TABLE VI.

Comparison of Single and Repeated Divided Doses of Drug, Given 1 Hour after Injection of the Organism.

Chemical.	Length of life.						
	After single dose of chemical.			After repeated doses of chemical, five times at hr. intervals.			
	Dose.*	No. of mice.	No. of M. L. D.*	Dose.*	No. of mice.	No. of M. L. D.*	
			10			10	20	50
	mg.			*mg.*				
C 29	0.5	4	24 hrs.; 9 days; 16 days; 24 days.	0.1	2	L.; L.†	144 hrs.; L.	L.; L.
C 36	1.0	4	60 " 72 hrs.; 36 hrs.; 120 hrs.	0.2	2	" "	L.; L.	" "
C 40	1.0	4	24 " 72 " 48 " 60 "	0.2	2	" "	" "	" "
C 110	2.0	4	72 " 240 " 96 " 168 "	0.4	2	" "	" "	" "
Optochin.	2.0	4	72 " 120 " 140 " L.	0.4	2	" "	48 hrs.; L.	48 hrs.; 48 hrs.
Controls.	0	6	0.00000001 cc., 48 hrs. = 1 M. L. D.	0	6	0.00000001 cc., 48 hrs. = 1 M. L. D.		

* Both drug and organisms were injected intraperitoneally.

† In the tables L. indicates lived.

TABLE VII.

Intraperitoneal Varying Doses of Drug Repeated Hourly for 5 Hours, Beginning 2 Hours after Injection of the Organism. Titration of Chemicals.

Chemical.	No. of M. L. D.	No. of mice.	Length of life after varying doses.			
			1.0 mg.	0.5 mg.	0.25 mg.	0.125 mg.
C 29	10	2		36 hrs.; 24 hrs.	36 hrs.; 36 hrs.	10 days; L.
	100	2		24 " 24 "	36 " 36 "	36 hrs.; 36 hrs.
C 36	10	2	24 hrs.; 24 hrs.	36 " L.	L.; L.	
	100	2	24 " 36 "	60 " "	36 hrs.; 96 hrs.	
C 40	10	2	24 " 24 "	36 " 60 hrs.	L.; L.	
	100	2	36 " 24 "	36 " 36 "	60 hrs.; L.	
C 110	10	2	L.; L.	L.; L.	L.; L.	
	100	2	36 hrs.; L.	60 hrs.; L.	36 hrs.; 36 hrs.	
Optochin.	10	2	L.; L.	7 days; L.	48 " 48 "	
	100	2	60 hrs.; L.	36 hrs.; 60 hrs.	36 " 36 "	
Controls.	0	4	Succumbed to 0.0000001 cc. = 1 M. L. D.			

TABLE VIII.

Intraperitoneal Doses of Drug Repeated Hourly for 5 Hours, Beginning 2 Hours after Injection of Varying Doses of the Organism.

Titration of Organisms.

Chemical.	Dose.	No. of mice.	Length of life.		
			No. of M. L. D.		
			10	20	40
	mg.				
C 29	1.25	4	L.; L.; L.; 96 hrs.	36 hrs.; 36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.; 36 hrs.
C 36	0.25	4	96 hrs.; 96 hrs.; 96 hrs.; 24 hrs.	L.; L.; L.; L.	36 " 36 " 36 " L.
C 40	0.25	4	L.; L.; L.; L.	96 hrs.; 36 hrs.; 36 hrs.; 36 hrs.	36 " 36 " 36 " 36 hrs.
C 110	0.5	4	36 hrs.; L.; L.; L.	36 " L.; L.; L.	36 " L.; 36 hrs.; L.
Optochin.	1.0	4	36 " " " "	L.; L.; L.; L.	36 " 36 hrs.; 96 hrs.; 96 hrs.

the 10 M.L.D. gave a sterile heart's blood culture, that of the 100 M.L.D. mouse being positive. All other mice of this experiment proved to have a septicemia at death. It can be seen with repeated, as with single injections, of C 29 and C 40, that there is a definite optimum concentration at which these drugs act; in addition, C 110, C 36, and optochin show this zonal phenomenon with repeated doses, C 110 and optochin having a broader zone than the other chemicals. Optochin, however, as in the preceding experiment, did not influence more than 10 M.L.D. when administered in small (0.25 mg.) repeated doses.

In the experiment represented in Table VIII, we estimated the number of minimum lethal doses of pneumococci killed by the optimum concentration of the drug. The results parallel those represented in Table VII, and in addition show that C 36 and C 110 and optochin influence as many as 20 M.L.D. The irregularity seen with C 36 has occurred in other experiments. Often with a small number of organisms, the infection proceeded as in the control mice, while a larger number of organisms was killed.

Diffusion of Drugs.

Thus far our work indicates that within certain definite limits these cinchona compounds have marked *in vivo* bactericidal activity. Drugs to be effective in an infectious disease should have more than this local action. They must be able to penetrate or diffuse throughout the animal body and perhaps in the last analysis maintain a definite concentration in the blood stream. This is especially true with the experimental infection of pneumococci in mice, which is a typical septicemia and in no way comparable to a lobar pneumonia. With this idea in view, the following experiments were carried out.

Subcutaneous Administration of Drugs Following Intraperitoneal and Intravenous Injection of Pneumococci.—This is the method which Morgenroth and Levy[1] and Moore[17] used in their investigations with optochin on mice. Their results show that optochin does diffuse into the circulation, inasmuch as an experimental infection was controlled in its incipiency.

Table IX is a record of three experiments in which the drug was administered subcutaneously, and the organism injected either intraperitoneally or intravenously. In the first experiment duplicate mice were given 1 and 10 M.L.D.

[17] Moore, H. F., *J. Exp. Med.*, 1915, xxii, 269, 389, 551.

TABLE IX.

Subcutaneous Injection of Drug.

Chemical.	Organism injected intraperitoneally.			Organism injected intraperitoneally.			Organism injected intravenously.		
	Dose.	No. of M. L. D.	Length of life.	Dose.	No. of M. L. D.	Length of life.	Dose.	No. of M. L. D.	Length of life.
	mg.			*mg.*			*mg.*		
C 29	0.5	1	L.; L.	2.0	1	48 hrs.; 48 hrs.	0.5	$\frac{1}{5}$	60 hrs.; 48 hrs.
		10	48 hrs.; 36 hrs.		10	48 " 48 "		1	48 " 48 "
C 36	1.0	1	L.; L.	4.0	1	36 " 48 "	1.0	$\frac{1}{5}$	48 " 96 "
		10	36 hrs.; 36 hrs.		10	36 " 48 "		1	48 " 48 "
C 40	1.0	1	L.; L.	2.0	1	L.; 48 hrs.	1.0	$\frac{1}{5}$	L.; L.
		10	36 hrs.; 48 hrs.		10	48 hrs.; 48 hrs.		1	60 hrs.; 60 hrs.
C 110	2.0	1	36 " 48 "	4.0	1	48 " 48 "	2.0	$\frac{1}{5}$	60 " 48 "
		10	36 " 36 "		10	48 " 48 "		1	60 " 60 "
Optochin.	2.0	1	L.; L.	4.0	1	L.; L.	2.0	$\frac{1}{5}$	L.; 60 hrs.
		10	48 hrs.; L.		10	" 96 hrs.		1	60 hrs.; 48 hrs.

of pneumococci intraperitoneally, followed immediately by the drug. There seem to be indications that the drugs, save C 110, do have a systemic distribution in sufficient quantity to influence the progress of the attack of virulent organisms. This action is slight, however, optochin having greater effectiveness than the other compounds. In the second experiment a large non-toxic dose was given to duplicate mice with 1 and 10 M.L.D. The infection was not influenced, except in the case of optochin. The same zonal phenomenon persisted that occurred with C 29 and C 40 and in fact all the aromatic compounds (Text-fig. 1); that is, a large non-toxic dose of the drug did not favorably influence the course of the infection, the mice, if anything, being more sick than those given smaller doses. The third experiment represents an attempt to influence the course of an experimental septicemia. Again in duplicate, mice were given $\frac{1}{5}$ and 1 M.L.D. (1:1,000 and 1:5,000) of a 6 hour culture of Neufeld pneumococci intravenously, followed immediately by the drug subcutaneously. It is seen that not only did the mice injected with 1 M.L.D. of the drug die, but all save those with C 40 and optochin of the $\frac{1}{5}$ M.L.D. series. Seemingly with this dose, at least, the natural defensive mechanism is interfered with, so that the animal receiving one-fifth of the number of pneumococci (approximately 40,000 organisms) necessary to cause the death of a mouse, died in the same time as the controls.

Intravenous Injection of Drug and Organism; Intravenous Injection of Organism and Intraperitoneal Injection of Drug, and Vice Versa.—If the subcutaneous injection of the drug decreased the ability of the animal to cope with virulent organisms, one might expect that injection of the drug intravenously would also destroy a part of this defensive process.

That this is the case is shown in the first experiment in Table X. Although optochin was not so toxic as the aromatic compounds, a single dose of the drug did not influence the progress of the infection with 1 M.L.D. of organisms. In the next experiment with the intravenous organism and intraperitoneal drug, the outcome was practically the same as with the intravenous injection of the organism and subcutaneous injection of the drug. Again not only does the intraperitoneal injection of the chemical not affect favorably an experimental septicemia in mice, but causes the animal to succumb to a number of organisms below the minimum lethal dose.

Diffusion of Drugs through the Gastrointestinal Tract.—Inasmuch as there has been found to be a definite therapeutic dose for these chemicals when given by the intraperitoneal and intravenous routes, varying amounts were employed to test their diffusibility from the gastrointestinal tract.

TABLE X.

Simultaneous Injection of Drug and Organism.

Chemical.	Both drug and organism injected intravenously.			Organism injected intravenously, drug intraperitoneally.			Organism injected intraperitoneally, drug intravenously.		
	Dose.	No. of M.L.D.		Dose.	No. of M.L.D.		Dose.	No. of M.L.D.	
		1/8	1		1/8	1		1/8	1
	mg.			*mg.*			*mg.*		
C 29	0.1	72 hrs.; 144 hrs.	60 hrs.; 11 days.	0.5	36 hrs.; 36 hrs.	36 hrs.; 36 hrs.	0.1	L.; L.	36 hrs.; 36 hrs.
C 36	0.2	48 " 120 "	36 " 36 hrs.	1.0	120 " 48 "	36 " 36 "	0.1	" 36 hrs.	36 " 36 "
C 40	0.1	36 " L.	36 " 7 days.	1.0	48 " 60 "	36 " 48 "	0.1	" L.	36 " 36 "
C 110	0.3	L.; L.	60 " 72 hrs.	2.0	L.; L.	60 " 72 "	0.3	" 36 hrs.	36 " 36 "
Optochin.	0.3	" "	120 " 48 "	2.0	" "	120 " 48 "	0.3	" 36 "	36 " 36 "

A series of four doses was used—both single and repeated—each being one-half the amount of the preceding (Table XI). Mice were injected intraperitoneally with 2 to 4 M.L.D. of organisms immediately followed by the drug *per os*. With the single dose there was demonstrated a diffusibility sufficient to influence this small number of pneumococci. As with the intraperitoneal injection of the drug, there is in this case also a concentration of chemicals above which the organisms are not killed, and the animal dies of septicemia. Although C 29 and C 110 delayed death, there were no actual survivals. C 36, C 40, and optochin did prevent the development of an infection, optochin, as before, being active in a large dose. On the other hand, repeated doses of all the drugs exerted a destructive influence upon the organism.

From this experiment it would be very difficult to say which one of the chemicals was the best. To determine this fact, the optimum concentration of the drug was used in another experiment with multiple lethal doses of pneumococci (Table XII).

Three doses of the chemical were given at 2 hour intervals, beginning immediately after the injection of the organisms. Although there was very little variation in effect of the different chemicals, results prove that inasmuch as there were some survivals, some action other than purely local was exerted. In the same table are presented the results concerning the effectiveness of drugs after intravenous injection of the organism. It happens that by this method of administration the same destructive action of the defensive mechanism of the animal does not occur with C 40, C 110, and optochin, as occurred by intraperitoneal route. In fact, with these compounds, there was a survival of the mice injected with 1 M.L.D. This experiment with intravenous injection of the organism was repeated, with multiple minimum lethal dose, but no more than 1 M.L.D. was influenced with C 40, C 110, and optochin, while with C 29 and C 36, death of the mice ensued on less than 1 M.L.D.

The rapidity with which the bactericidal activity of these compounds is neutralized within the animal body is shown in Table XIII.

A therapeutic dose of each drug was injected into the peritoneal cavity of mice, in triplicate, and 5 minutes later, 1, 10, and 20 M.L.D. of pneumococci were injected by the same route. An amount of chemical which in simultaneous injections kills 500 to 8,000 M.L.D. is fixed, changed, exhausted, or somehow destroyed in 5 minutes, so that all the mice injected with 10 M.L.D. died, and none receiving 20 M.L.D. survived.

TABLE XI.

Per Os Therapy after Intraperitoneal Injection of 2 M.L.D. *of the Organism.*

Chemical.	Single doses immediately after injection of organism.			Three doses, 2 hrs. apart; first immediately after injection of organism.		
	Dose.	No. of mice.	Length of life.	Dose.	No. of mice.	Length of life.
	mg.			*mg.*		
C 29	2.5	4	48 hrs.; 72 hrs.; 72 hrs.; 120 hrs.	2.5	3	24 hrs.; 24 hrs.; 48 hrs.
	1.25	3	48 " 48 " 96 "	1.25	3	24 " 24 " L.
	0.625	3	72 " 48 " 48 "	0.625	3	48 " 72 " "
				0.25	3	48 " L.; L.
C 36	5.0	4	48 hrs.; L.; L.; L.	5.0	3	24 " 24 hrs.; 36 hrs.
	2.5	3	72 " " "	2.5	3	24 " 48 " L.
	1.25	3	72 " " "	1.25	3	48 " L.; L.
				0.625	3	54 " " "
C 40	5.0	4	48 hrs.; 48 hrs.; 48 hrs.; 54 hrs.	5.0	3	24 " 48 hrs. 48 hrs.
	2.5	3	L.; L.; L.	2.5	3	24 " 48 " 48 "
	1.25	3	72 hrs.; L.; L.	1.25	3	48 " 48 " L.
				0.625	3	L.; L.; L.
C 110	5.0	4	48 hrs. 60 hrs.; 72 hrs.; 72 hrs.	5.0	3	24 hrs.; 24 hrs.; 48 hrs.
	2.5	3	48 " 96 " 72 "	2.5	3	24 " 48 " 60 "
	1.25	3	48 " 72 " 72 "	1.25	3	48 " L.; L.
				0.625	3	72 " " "
Optochin.	5.0	4	72 hrs.; L.; L.; L.	5.0	3	48 hrs.; 48 hrs.; 48 hrs.
	2.5	3	72 " 72 hrs.; 72 hrs.	2.5	3	48 " 72 " L.
	1.25	3	48 " 72 " 72 "	1.25	3	48 " L.; L.
				0.625	3	48 " 48 hrs.; L.

TABLE XII.

Per Os Therapy. Three Doses Repeated at 2 Hour Intervals; First Dose Simultaneous with Injection of the Organism.

Chemical.	Dose.	No. of mice.	Length of life. No. of M. L. D. injected intravenously. ½	Length of life. No. of M. L. D. injected intravenously. 1	Dose.	No. of mice.	Length of life. No. of M. L. D. injected intraperitoneally. 2	Length of life. No. of M. L. D. injected intraperitoneally. 20	Length of life. No. of M. L. D. injected intraperitoneally. 40
	mg.				*mg.*				
C 29	0.25	4	24 hrs.; 24 hrs.; 48 " 6 days.	24 hrs.; 48 hrs.; 48 " 72 "	0.25	4	48 hrs.; 48 hrs.; L.; L.	96 hrs.; L.; L.; L.	48 hrs.; 72 hrs.; 72 " 72 "
C 36	0.5	4	36 " 36 hrs.; 4 days; 6 days.	36 " 48 " 96 " 6 days.	0.5	4	48 hrs.; 72 hrs.; L.; L.	48 hrs.; 48 hrs.; L.; L.	48 " 72 " 72 " L.
C 40	0.5	4	L.; L.; L.; L.	36 " 48 hrs. L.; L.	0.5	4	L.; L.; L.; L.	72 hrs.; L.; L.; L.	48 " 72 hrs.; 6 days; L.
C 110	1.0	4	" " " "	L.; L.; L.; L.	1.0	4	48 hrs.; L.; L.; L.	48 hrs.; L.; L.; L.	48 hrs.; 48 hrs.; L.; L.
Optochin.	1.0	4	" " " "	" " " "	1.0	4	48 hrs.; L.; L.; L.	48 hrs.; L.; L.; L.	48 hrs.; 48 hrs.; 48 " L.
Controls.	0	4	0.0005 cc. 6 days; L. L.; L.	0.0001 cc. 36 hrs.; 48 hrs.; 96 hrs.; 120 hrs.	0	4	48 hrs.; 48 hrs.; 48 " 48 "	36 hrs.; 36 hrs.; 36 " 36 "	36 hrs.; 36 hrs.; 36 " 36 "

TABLE XIII.

Protection Experiment. Drug Injected 5 Minutes before the Organism.

Chemical.	Dose.	No. of mice.	Length of life. No. of M.L.D. 1	10	20
	mg.				
C 29	0.5	3	36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.
C 36	1.0	3	12 " 72 " 36 "	36 " 36 " 36 "	60 " 36 " 36 "
C 40	1.0	3	36 " L.; L.	36 " 48 " 240 "	36 " 36 " 36 "
C 110	2.0	3	36 " " "	36 " 72 " 120 "	36 " 72 " 72 "
Optochin.	2.0	3	48 " " "	36 " 72 " 72 "	36 " 60 " 72 "
Controls.	0	3	36 " 36 hrs.; 36 hrs.	36 " 36 " 36 "	36 " 36 " 36 "

Our work as so far reported has been restricted to a Type I pneumococcus (Neufeld). Moore[2] showed that there is a difference in the bactericidal action of optochin with pneumococci of the different types, but it is not necessarily specific for types. Using the same procedure of simultaneous intraperitoneal injection of organism and drug, described above in connection with the Neufeld pneumococcus, we have titrated an additional Type I and two each of Types II and III (Table XIV). It would appear from these results that the chemicals are most active against Type I, less so against Type II, and least against Type III. That this specificity perhaps is not as

TABLE XIV.

Comparison of the Relative Protection Afforded by Different Drugs against Multiple Lethal Doses of Type I, II, and III Pneumococci. Simultaneous Injection Intraperitoneally of Drug and Organism.

Chemical.	No. of M. L. D.					
	Type I.		Type II.		Type III.	
	Neufeld.	No. 15.	No. 28.	No. 32.	No. 14.	No. 27.
C 29	500	100	400	20	0	1
C 36	5,000	1,000	400	50	0	0
C 40	8,000	1,000	500	100	0	1
C 110	6,000	1,000	400	50	10	1
Optochin.	700	400	400	50	10	1
No of organisms per M.L.D.	4	18	9	8	700,000	5,000,000

marked as it would seem is borne out, even in the case of these six strains, by two considerations. First, the virulence of the Type III strains was not as great as that of either of the Type I or of the Type II, the Type III having a minimum lethal dose in one strain of 700,000 and in the other of 5,000,000 organisms. It is possible that we are dealing not with type specificity but with organisms more or less resistant to chemicals and of varying numerical degrees of virulence. This is emphasized in the experiment shown in Table XV. Then, during another investigation, the virulence of the Type I (Neufeld) was reduced, so that 1 M.L.D. contained 7,000 instead of two to four organisms. This organism was used in multiple lethal doses, 1, 10, 100, and

TABLE XV.

Effect of Drugs on a Neufeld Type I Pneumococcus of Lowered Virulence (Decreased Approximately 4,000 Times).

Chemical.	Dose.	No. of mice.	Length of life.			
			No. of M.L.D.			
			1,000	100	10	1
			No. of organisms.			
			6,500,000	650,000	65,000	7,000
	mg.					
C 29	0.5	2	48 hrs.; 48 hrs.	48 hrs.; 48 hrs.	96 hrs.; 112 hrs.	L.; L.
C 36	2.0	2	48 " 48 "	48 " 48 "	48 " 96 "	" "
C 40	1.0	2	48 " 48 "	48 " 48 "	L.; L.	" "
C 110	2.0	2	48 " 48 "	48 " 48 "	48 hrs.; L.	" "
Optochin.	2.0	2	48 " 48 "	48 " 48 "	48 " 48 hrs.	48 hrs.; 96 hrs.
Controls.	0	2	48 " 48 "	48 " 48 "	48 " 48 "	48 " 48 "

1,000, and followed immediately by the chemical (simultaneous method). The results with organism in this stage of activity were very similar to those with Type III above, the chemicals, except C 40, being active against not more than 1 M.L.D.

Rabbit Experiment.

Two experiments were carried out with rabbits (Table XVI). Organisms and drugs were injected simultaneously. Regardless of toxicity, 25 mg. per kilo of each chemical were used. The pneumococcus (Neufeld) in a preliminary titration demonstrated a virulence such that 0.000001 cc. of a 6 hour serum broth culture

TABLE XVI.

Rabbit Experiment.

Chemical.	Pneumococci 0.00001 cc.					Pneumococci 0.000001 cc.				
	Dose per kilo.	Rabbit No.	Weight.	Life.	Culture.	Dose per kilo.	Rabbit No.	Weight.	Life.	Culture.
	mg.		*gm.*	*days*		*mg.*		*gm.*	*days*	
C 29	25	1	2,300	3	++	25	9	2,100	4	++
C 36	25	2	1,900	11	++	25	10	1,775	L.	
C 40	25	3	1,540	L.		25	11	2,000	"	
C 110	25	4	1,850	7	++	25	12	2,020	"	
Optochin.	25	5	1,950	2	++	25	13	1,675	3	++
	25	6	2,000	3	++	25	14	2,200	3	++
Controls.	0	7	1,800	3	++	0	15	1,950	3	++
	0	8	2,100	3	++	0	16	2,100	3	++

killed a 2 kilo rabbit in 3 days. In the first experiment with 0.0001 cc., or 100 M.L.D., the rabbit treated with C 40 remained alive (30 days), those treated with C 36 and C 110 showed a slight delay, while the controls, the C 29, and the optochin animals succumbed in 2 or 3 days. In the second experiment the same procedure was carried out, except that 1 cc. of 0.00001 dilution of organisms was employed for injection. The C 29, optochin, and control animals died in 4, 3, and 3 days respectively, those of C 36, C 40, and C 110 survived.

These results show that the bactericidal activity of the aromatic compounds is not restricted to mice. Optochin seemingly does not act with sufficient rapidity in rabbits to kill this number of organisms *in vivo*. The results of this experiment confirm the work with optochin of Moore,[17] Scott,[18] and Lewis.[19]

[18] Scott, W. M., *J. Path. and Bact.*, 1914–15, xix, 130.

[19] Lewis, J. H., *Arch. Int. Med.*, 1918, xxii, 593.

DISCUSSION.

The aromatic compounds described are a series of chemicals possessing rapid bactericidal action both *in vitro* and *in vivo*. There is, however, a pronounced variation in their action from the bacteriotropic and organotropic standpoint. C 29 ranks first in rapidity of action *in vitro*, but because of its polytropic characteristics is the poorest *in vivo*. The para-hydroxy substitution product (C 36) is an improvement in that the decrease in bactericidal action is more than compensated for by the lessening of its toxicity for mice. The bactericidal activity *in vitro* of the meta-hydroxy compound (C 40) is almost as great as the unsubstituted benzene derivative (C 29), and its toxicity is reduced 50 per cent. The dihydroxybenzene compound (C 110), judged by its ability to kill multiple lethal doses of pneumococci *in vivo*, holds an intermediate position between the para and meta derivatives. Its *in vitro* bacteriotropic activity, however, is much less than that of both the other aromatic chemicals and optochin. This substance is an example of a drug which, when injected simultaneously with multiple lethal doses of a virulent pneumococcus into the peritoneal cavity of mice, causes the death of a greater number of organisms than a corresponding amount *in vitro*. Apparently in a therapeutic dose its activity is auxiliary or perhaps stimulatory to the other natural forces of an animal which combat an infection. From the above it may be seen that there is a relationship between chemical constitution and chemotherapeutic activity.

The ultimate test of a chemotherapeutic agent is whether its use results in the cure of an established infection. Mice are unsatisfactory animals with which to make this test. The incubation period for the pneumococcus is so short, as well as the length of life following a fatal dose of the organism, that any remedial measure, if treatment be delayed till the infection is systemic in nature, may give misleading results.

Our method of investigation has shown that when pneumococci and drugs are injected simultaneously into the peritoneal cavity the death of multiple lethal doses of the organisms results. Any delay in the administration of the drugs neutralizes their effectiveness. Although an experimental infection was arrested when treatment was

postponed 2 hours after the injection of multiple lethal doses of organism (with chemicals and pneumococci injected intraperitoneally), at no time have we been able to cure a mouse having a systemic infection. The treatment involving the longest delay was administered after an 8 hour interval. In this experiment, not reported above, mice were injected with 1 M.L.D. and treatment was begun 8 hours later. 60 per cent of the mice treated with the aromatic compounds, with the exception of C 29, survived. Inasmuch, however, as pneumococci did not appear in the blood stream of the controls until 12 hours had elapsed, this study merely proves the chemicals to have a local therapeutic action, though of a high degree of efficiency. However, that there is some therapeutic activity resulting from diffusion of the drugs, as tested by injecting chemicals and organisms through different routes, is shown by the favorable results obtained in *per os* therapy.

Binz,[20] in an extensive series of experiments, has shown that, along with other physiological and pharmacological attributes of certain of the cinchona alkaloids, (1) they are general protoplasmic poisons, (2) stimulating in small amounts the activity of ameba, Infusoria, and white blood cells, in larger amounts paralyzing, and (3) decreasing the oxidizing and reducing power of the blood and weakening the enzymotic action. The summary of his work indicates that quinine in small amounts stimulates normal functional activity, in large amounts causing paralysis of this same mechanism. We are unable to say that these aromatic derivatives produce the same effect, but it has been shown that in experiments with pneumococcus infection of mice their action is zonal; that is, within certain dosage the bacteriotropic action of the chemicals is greater than the deleterious influence on the defensive mechanism of the animal. Amounts above this therapeutic dose, although non-lethal, paralyze processes vital for the animal's defense, permitting the invading organism to develop without restraint. It is difficult to understand why this large, non-lethal dose does not kill the bacteria, when seemingly more than a sufficient quantity to do so is injected. There is an apparent reversal of relationship from bacteriotropism with small doses to organotropism with the large. In other words, there is a greater affinity for the animal

[20] Binz, C., *Virchows Arch. path. Anat.* 1869, xlvi, 67; Das Chinin. Nach den neuern pharmakologischen Arbeiten dargestellt, Berlin, 1875.

tissue than for the pneumococci, when large amounts of chemical are injected. This zonal phenomenon was found to occur in intraperitoneal, subcutaneous, and *per os* administration of all the drugs, optochin having a less pronounced zonal effect than the aromatic compounds.

The question arises as to whether it would be advisable to use drugs for chemotherapeutic purpose that present this zonal phenomenon, which at first glance seems so discouraging. That this may not be an insuperable barrier is suggested by the fact that it is present in some degree in all drugs used in clinical medicine. Drugs in general have a therapeutic zone beyond which treatment cannot be carried with safety to the patient. The practical value of any therapeutic agent lies in its ability to produce desired effects in relatively nontoxic quantities.

The question naturally arises whether the aromatic cinchona compounds which are reported in this paper may have therapeutic applications in man. This cannot be answered until further and wider studies have been made. Our experiments have been confined almost entirely to the one animal species; but that other larger animals can be used may be inferred from the experiments successfully carried out on rabbits. That the list of synthesized pharmacological agents effective against a bacterial infection has been enlarged is obvious. But we feel that the time for the employment of any of the drugs here described in man has not yet arrived. Further investigations along the lines followed may not impossibly lead to the goal being sought.

CONCLUSION.

It has been shown with one strain of pneumococcus (Type I, Neufeld), that hydroquinine chloroacetanilide (C 29), hydroquinine *p*-chloroacetylaminophenol hydrochloride (C 36), hydroquinine *m*-chloroacetylaminophenol hydrochloride (C 40), and hyroquinine 4-chloroacetylaminopyrocathecol hydrochloride (C 110) have a rapid pneumococcidal activity both *in vitro* and in the peritoneal cavity of mice, and to a lesser extent in rabbits. In comparison, optochin is slower in action, but its power is not so easily destroyed either *in vitro* or *in vivo*.

The introduction of the hydroxy group of the benzene nucleus of hydroquinine chloroacetanilide changes the relationship between

organotropism and bacteriotropism. In comparing the rapidity of *in vitro* bactericidal action and intraperitoneal toxicity, C 29 exhibits the most rapid pneumococcidal action and is the most toxic for mice. C 36, the para-hydroxy derivative, is one-fifth as toxic as C 29 and only one-tenth less active bactericidally. C 40 is one-half as toxic and has approximately the same bactericidal power, while C 110 is one-eigth as toxic and one-fifth as pneumococcidal; and optochin is one-sixth as toxic and has one-fifth the bactericidal action. Arranged in the order of their ability to kill pneumococci when injected simultaneously with them into the peritoneal cavity, the drugs are: C 40, C 110, C 36, optochin, and C 29.

The chemotherapeutic action of the aromatic compounds is essentially local in character. But by *per os* therapy there is demonstrated a certain amount of diffusion of this activity, not shown by any other method of administration, C 40 and C 110 having about the same value as optochin.

Intravenous injection of the drugs in small doses destroyed to a greater or less extent the natural defenses of the animal, optochin being perhaps less injurious than the aromatic compounds. This same destruction of natural resistance followed intraperitoneal and subcutaneous injections of the chemicals as measured by intravenous injections of the organisms.

The maximum tolerant dose in a single injection (intraperitoneal) is not so efficacious as the same dose divided in fifths and injected at hour intervals. Optochin under these conditions is not so active as the aromatic compounds. In general, repeated doses are more curative than single.

There is a zone between the therapeutic and toxic doses, both single and repeated, for all these chemicals alike, where the natural resistance of the animal to an infection is reduced. This effect is noted especially with C 29, C 36, and C 40. In the case of optochin the therapeutic dose is nearer the toxic than with C 110, C 36, and C 40. Apparently these chemicals exhibit a variability in *in vivo* bactericidal activity according to different strains of pneumococci and numerical virulence.

We wish to thank Dr. W. A. Jacobs and Dr. M. Heidelberger for the supply of chemicals and other assistance.

A FURTHER INQUIRY INTO THE SOURCE OF THE VIRUS IN BLACKHEAD OF TURKEYS, TOGETHER WITH OBSERVATIONS ON THE ADMINISTRATION OF IPECAC AND OF SULFUR.

By ERNEST EDWARD TYZZER, M.D., and MARSHAL FABYAN, M.D.

(*From the Department of Comparative Pathology of Harvard Medical School, Boston.*)

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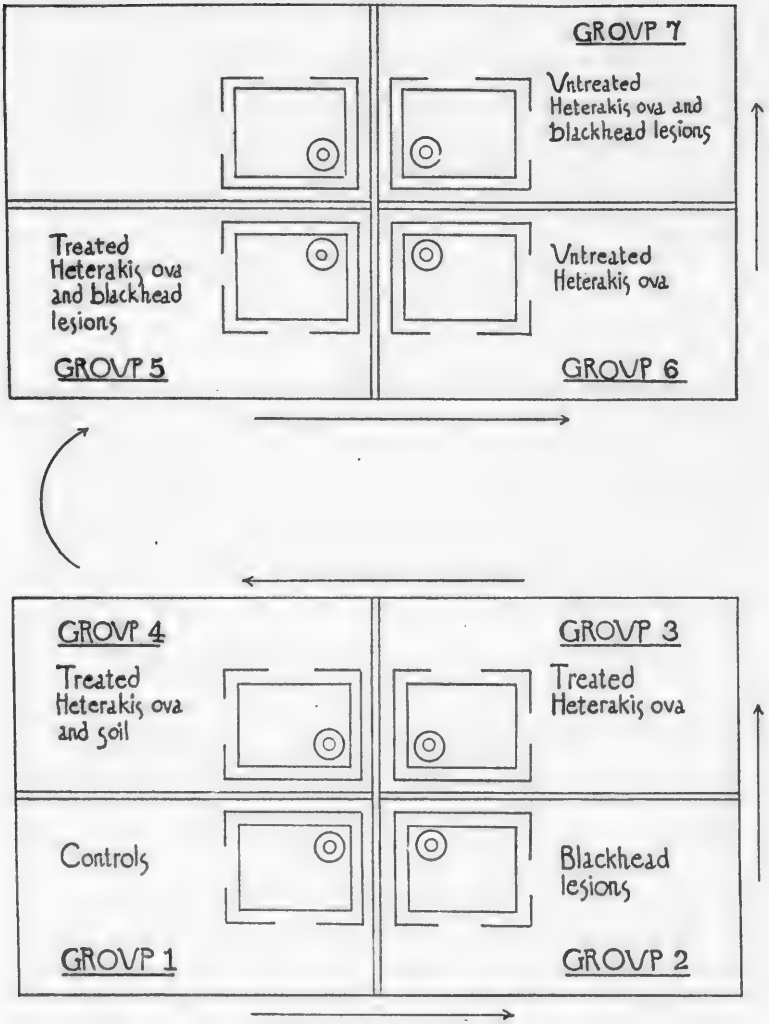
The investigation of blackhead in turkeys has been continued during the past season with two main objects in view: first, to obtain further evidence concerning the source of the virus in the natural transmission of the disease; and second, to test the efficiency of certain drugs in the prevention or treatment of this infection. Other observations with respect to contraction of the disease from the soil, especially that of hen yards, the occurrence of the disease in chickens, and the immunity following recovery from infection will also be discussed.

The Source of the Virus in Natural Transmission.

The following experiment was planned to determine if possible the source of the protozoan parasite of blackhead, *Histomonas meleagridis*, and more especially whether, in the natural transmission of blackhead, it is derived from the intestinal worm, *Heterakis papillosa*. A series of thirty-one turkeys from a single incubation was used in the experiment. These were divided into seven groups on removal from the incubator, each group being provided with a separate clean compartment in a laboratory room. They all received sterilized food and water, and were otherwise protected from any protozoan contamination throughout the experiment. The general arrangements of the compartments in which the turkeys were kept is shown in Text-fig. 1, and, as the value of the experiment lay in attention to minute details to remove any question of outside contamination, the outfit used will be described rather fully.



TEXT-FIG. 1. Plan of the compartments showing the distribution and treatment of the seven groups of turkeys used in Experiment 1. The arrows indicate the sequence in which the groups were fed and attended.

The compartments, which were also used in later experiments, were constructed by building intersecting partitions of wallboard on the tops of two large tables and an enclosing wall consisting of a double strip of cheese-cloth, fastened to posts at the four corners of each table top and to the ends of the partitions. In order to eliminate flies and other insects, a mosquito bar was stretched over the top and tucked inside the cheese-cloth strip. The legs of the tables were placed in dishes of kerosene oil to isolate the compartments from any crawling insects. A simple hover was provided in each compartment with a 15 watt electric light enclosed in a tin can to furnish heat. The hovers consisted of rectangular frames with legs at the four corners, across which were stretched wires and over these were hung strips of heavy cotton flannel. The top was covered with the same material. In order to retain the heat, a pasteboard box was placed over each hover and openings were made on two sides for the turkeys to go in and out. It was necessary to see that the turkeys were all safely under the hovers at the end of the 1st day after removal from the incubator but thereafter no further attention in this respect was required.

Before each experiment, the mosquito bar, cheese-cloth, hovers, and cans were autoclaved, the partitions given a fresh coat of paint, and the table tops covered with many thicknesses of fresh clean paper. New boxes were provided for the hovers. Drinking fountains and food dishes were either autoclaved or sterilized by boiling. All materials entering into the diet were sterilized. Each compartment was furnished with a litter of sterile hayseed. Hard boiled eggs were fed throughout the greater part of the experiment. Sterilized grit was kept constantly on hand and after a few days pasteurized milk, soured by inoculation with a stock culture of *B. bulgaricus*, and sterilized chick feed were added to the diet.

The attendant was required to wash her hands thoroughly with soap and water and then in 70 per cent alcohol before feeding the turkeys. The food was dropped or poured into each compartment and, in case anything was touched, the hands were rinsed in alcohol before proceeding to the next compartment. Any dish removed from the compartment was sterilized before it was returned.

The above precautions were taken in order to eliminate any possibility of the introduction of *Histomonas* except in the additional materials which were intentionally furnished to the various groups.

Experiment 1.—Thirty-one turkeys hatched on June 2, 1921, were removed from the incubator the following day, divided into seven lots or groups, each lot being placed in a separate compartment and furnished with sterilized food and water as described above.

When 4 days old, the turkeys of six groups were given their first dose of the various materials, the infectiousness of which was to be tested. Group 1 received nothing additional and served as controls; Group 2 was fed liver lesions from acute blackhead cases; Group 3 received ripe *Helerakis* ova that had been treated for 3 days with 1.5 per cent by volume of nitric acid; Group 4 received similarly treated *Heterakis* ova and was also given a sod of clean lawn grass; Group 5 received both the treated *Heterakis* ova and liver lesions; Group 6 was

fed untreated *Heterakis* ova only; and Group 7 received untreated *Heterakis* ova and liver lesions. These various materials were fed on a number of occasions, the dates of which are given in Table I. The groups were at all times fed in numerical order beginning with the controls (Text-fig. 1). This sequence in feeding was adopted intentionally to lessen the chance of contamination from any possible carelessness in carrying out directions. The attendant thus first fed the normal birds, then those which might possibly develop blackhead, and finally those which according to previous results would be most likely to develop the disease.

As each turkey died or was killed, a thorough postmortem examination was made, and the cecal contents were carefully examined for both worms and protozoa. The data of the experiment are furnished in detail in Table I.

That the measures taken to prevent accidental contamination of the stock employed in this experiment were adequate is shown by the postmortem findings in the group of control turkeys which was kept under the conditions already outlined for periods longer on the average than those in any of the other groups. None of the control turkeys showed any lesion, intestinal worm, or any form of protozoon.

The second group, which had been fed liver lesions fresh from cases of acute blackhead on three separate occasions, furnished two cases (50 per cent) of blackhead. One of these turkeys (No. 20), killed 15 days after the first feeding, showed typical blackhead; the other (No. 18), killed 21 days after the first feeding, showed a distribution of lesions that had hitherto never been encountered. As both ceca appeared to be normal throughout, presumably a large lesion of the proventriculus represented the primary focus, and smaller lesions in the liver metastases. Contact lesions resulting from the extension of the disease from liver lesions to the contiguous serous surface of the proventriculus are not infrequent, but in this case there was extensive necrosis of the mucosa without any visible involvement of the serous coat. A coccidium (*Eimeria avium*) was also found in three of the four turkeys of this group. Whether this very resistant organism was derived from some of the turkey eggs from which the birds were hatched, or from the diseased livers which were fed to them, or from air-borne dust, could not be determined. It should be mentioned that, of the various compartments, the one containing these birds was nearest a window (8 feet) which was kept open more or less all the time for ventilation. While this is suggestive of air-

borne infection the other possibilities are quite as plausible. The important point, however, is that the feeding of considerable amounts of blackhead virus may of itself produce infection in the absence of *Heterakis papillosa* or other parasitic worms.

The development of blackhead in all the turkeys of the third group furnishes very suggestive evidence as to the origin of the virus in the natural transmission of the disease. The previous treatment of the *Heterakis* material, *i.e.* subjection for 3 days to the action of 1.5 per cent nitric acid, rendered this material sterile with respect to bacteria (as shown by cultures) so that the survival of the protozoon, *Histomonas meleagridis*, outside of the *Heterakis* ova in this material is extremely improbable. This material, however, did produce typical blackhead in fatal form in each of the five turkeys to which it was fed, and the symptoms of the disease appeared as early as in the cases in which untreated *Heterakis* material was employed.

In case the virus was external to the membrane which so adequately protects the *Heterakis* embryos in the ripe eggs of this worm, it would be reasonable to expect at least a dilution effect as the result of the treatment with acid. Even if it were conceivable that the albuminous matter present did furnish a certain amount of protection, the frequent agitation in a large amount of acid and the subsequent washings in sterile salt solution would tend to eliminate any virus not included in the ova. All previous experiments indicate that the organism of blackhead is of a frail, non-resistant character. Inoculation is successful only when freshly obtained lesions are used, although at a low temperature (5°C.) the latter may remain active at least 4 days. Although the organisms may be maintained in an active state for a few days in appropriate culture media, they soon disappear completely, leaving no forms to be interpreted as resistant stages.

The possibility that the parasite of blackhead is transmitted through the turkey's egg to the young turkey is not wholly eliminated. However, if such were the case one would at least expect the disease to appear occasionally in isolated control turkeys, especially in those not rare instances in which pathological conditions as indicated by fibrinous cores are present in the ceca of otherwise healthy birds.

The demonstration that the feeding of the acid-treated *Heterakis* ova is all that is necessary for the production of blackhead leaves the

TABLE I.

Group.	Turkey No.*	Treatment.	Death.†	Anatomical findings.	*Heterakis.*‡	Protozoa.
			1921			
1 Controls.	11	None, other than isolation and a diet of sterilized food.	June 21. K.	Normal.	0	None.
	12		" 30. "	"	0	"
	13		" 30. "	"	0	"
	14		" 25. "	"	0	"
	15		" 30. "	"	0	"
	16		" 27. "	"	0	"
2 Fed blackhead lesions.	17	June 6, 8, and 18, 1921. Fed fresh liver lesions.	June 27. K.	Normal.	0	*Eimeria avium.*
	18		" 27. "	Atypical blackhead, primary in proventriculus, secondary in liver.	0	*Histomonas* and *Eimeria avium.*
	19		" 28. "	Normal.	0	*Eimeria avium.*
	20		" 21. "	Typical blackhead.	0	*Histomonas.*
3 Fed treated *Heterakis* ova.	21	June 6 and 9, 1921. Fed *Heterakis* ova treated for 3 days with 1.5 per cent nitric acid.	June 24. D.	Typical blackhead.	21	*Histomonas.*
	22		" 23. K	" "	36	"
	23		" 25. D.	" "	7	"
	24		" 25. K.	" "	3	"
	25		" 28. D.	" "	9	"
4 Fed treated *Heterakis* ova; soil.	26	June 6 and 9, 1921. Fed *Heterakis* ova treated for 3 days with 1.5 per cent nitric acid.	June 24. D.	Typical blackhead.	7	*Histomonas.*
	27		" 27. "	" "	9	"
	28		" 28. K.	" "	2	" (pulsating forms).
	29	Given sod from lawn.	" 22. "	" "	20	"

5 Fed treated *Heterakis* ova and blackhead lesions.	30 31 32 33	June 6 and 9, 1921. Fed *Heterakis* ova treated for 3 days with 1.5 per cent nitric acid. June 6, 8, and 18. Fed fresh liver lesions.	June 27. K. " 28. D. " 27. " " 7. K.	Typical blackhead. " " " " Cecal but no liver lesions.	12+§ 70+§ 17 6	*Histomonas.* " " "
6 Fed untreated *Heterakis* ova.	34 35 36 37	June 6 and 9, 1921. Fed untreated *Heterakis* ova.	June 25. D. " 25. " " 28. " " 24. K.	Typical blackhead. " " " " " "	28 19 5‡ 24	*Histomonas.* " " "
7 Fed untreated *Heterakis* ova and blackhead lesions.	38 39 40 41	June 6 and 9, 1921. Fed untreated *Heterakis* ova. June 6, 8, and 18. Fed fresh liver lesions.	June 24. K. " 28. " " 28. D. " 28. K.	Typical blackhead. " " " " " "	132+§ 5 11‡ 15	*Histomonas.* " (few pulsating forms). *Histomonas.* " (numerous pulsating forms).

* Turkeys hatched June 2, 1921.

† K. indicates killed; D., died.

‡ In the *Heterakis* column + indicates worms present; ++ worms in moderate numbers; +++ worms numerous; in combination with a number + indicates worms present in excess of that number.

§ Microscopic larvæ also noted.

additional factors introduced in the four remaining groups without significance. Thus it is unimportant whether soil, virus derived from blackhead lesions, or fecal material is present with *Heterakis* ova, for the result in all cases is the same. The sixteen turkeys of these various groups all showed typical blackhead.

The groups fed acid-treated ova showed somewhat fewer *Heterakis*, visible to the naked eye (average 17), as compared with the groups fed untreated ova (average 30), but the individual distribution within the groups varied greatly, one turkey of a given group showing only 5, another 132 worms.

The finding of *Heterakis* in various stages of development, and especially the occurrence of small larval forms, weeks after the *Heterakis* material was fed, indicates that many of the ova pass through the alimentary tract of the young turkey intact and that these, on being again picked up from the litter in which food becomes scattered, may then hatch and develop.

In the ceca of several of the turkeys which survived longest in this experiment, rounded protozoa having the general appearance of small sized *Histomonas* were observed to pulsate rhythmically though weakly. This observation was suggestive of the occurrence, late in the infection, of a flagellated stage of *Histomonas*, but the point could not be definitely determined at this time because pulsating forms were found only in those turkeys which had been fed untreated *Heterakis* material or had been furnished soil.

The young turkeys were undoubtedly infected long before they showed any pronounced symptoms. Possibly their confinement in a small space in which they were not subjected to the tiring effects of a more variable environment, such as they experience when allowed to roam free, was the cause of their surviving much longer than would be expected in view of their age.

Experiment 2.—A lot of eleven turkeys hatched on September 6, 1921, was kept in a clean compartment and furnished sterilized food and water. One found dead at the age of 5 days showed firm slender cores in the ceca without any thickening of the walls; two died at the age of 8 days, one being crop-bound, and the other showing a firm yellow core distending one cecum. In none of these was there any indication of blackhead infection. On September 15, two were moved to another clean compartment and during the next 24 hours were fed five doses of

ripe *Heterakis* ova (collected in August) that had been kept for a week in 1.5 per cent nitric acid. The six other turkeys of this lot were fed a portion of the same *Heterakis* material which had not been treated (two on September 15 and four on September 22).

Both turkeys receiving the *Heterakis* ova kept for a week in dilute nitric acid developed characteristic blackhead. One was killed 21 days and the other died 28 days after receiving the last dose of ova. They both showed few *Heterakis* of sufficient size to be seen with the unaided eye but scrapings of the cecal mucosa showed numerous young larvæ.[1]

The turkey which died 28 days after being fed acid-treated *Heterakis* material was autopsied at once and showed in the ceca numerous motile flagellates, apparently representing a flagellated stage of *Histomonas*. No other protozoa were found in fresh preparation, stained film, or section. In scrapings of the cecal mucosa planted in ovomucoid, a medium in which *Tetratrichomonas gallinarum* is readily grown, the above organisms died out completely within 48 hours. In stained films the organisms were readily identified as *Histomonas meleagridis* and in a considerable proportion one or two short flagella were demonstrable.

With the view of obtaining early stages of blackhead for histological studies, two of the turkeys that received untreated *Heterakis* material were killed 8 days later, one 9 days, two 10 days, and one 11 days after the ingestion of ova. In one a single *Heterakis* larva was found, one showed four, and the others numerous young worms. Blackhead was found in only one of the turkeys, killed 10 days after ingestion of ova.

The results of this experiment confirm those of Experiment 1 with respect to production of blackhead with *Heterakis* ova treated with dilute (1.5 per cent) nitric acid. They also show that *Histomonas meleagridis* may occur in flagellated form late in the disease. Small *Heterakis* larvæ were again found long after feeding the ripe ova of this worm.

That only one of the six turkeys that were fed untreated ova and killed from 8 to 11 days later should show blackhead infection indicates that invasion of the tissues by the organism of blackhead does not always immediately follow the ingestion of the worm eggs. From the results of previous experiments it appears probable that others of this lot would have become infected if they had lived 1 or 2 weeks longer.

[1] Some of these larvæ were no larger or more differentiated than the stages obtained 5 days after feeding *Heterakis* ova by Dr. C. Uribe of this laboratory who is making a study of the life cycle of this worm

The Effect of Drugs on Blackhead in Turkeys.

Ipecac Treatment.—In the course of a previous investigation[2] emetine, the active principle of ipecac commonly employed in amebic dysentery, was administered at regular intervals in toxic doses to two turkeys inoculated with blackhead. This drug failed to prevent the development of the subcutaneous lesions in either case. While one of the turkeys eventually recovered it was not thought that this should be attributed to the emetine, since spontaneous recovery from inoculated blackhead had already been noted. In a series of nine turkeys infected with blackhead through inoculation during the present investigations, three have recovered without treatment.

The recent report of Wegeforth[3] on the value of ipecac in the prevention and treatment of blackhead in turkeys has aroused widespread interest. It appeared quite possible that ipecac, as it contains active principles other than emetine, might be of value even though the latter had failed. Furthermore, a drug which failed to destroy organisms injected into the tissues might prevent natural invasion from the cecal contents especially if administered by mouth. It was to test the value of ipecac that the following experiments were planned.

Experiment 3.—Ripe *Heterakis* ova were fed on July 20, 1921, to fifteen of a lot of forty turkeys hatched on July 14. Wine of ipecac was then administered to four of these in doses of 5 drops daily to see if infection could be prevented.

The failure of these ipecac-treated turkeys to contract blackhead is of doubtful significance, for only two of the eleven control turkeys showed blackhead infection. The absence of worms in four of the latter and the small number found in others indicated that the dosage of *Heterakis* ova was too small to furnish uniform results. 23 days after the feeding of *Heterakis* ova one of the four ipecac-treated turkeys was killed and showed no evidence of disease or intestinal worms. The other three were used in the following experiment.

Experiment 4.—Twenty-three turkeys hatched on July 14, 1921, were used, three having previously received small daily doses of wine of ipecac as already explained. On August 11 the turkeys, now 28 days old, were placed out of doors in two adjacent cages, twelve in one and eleven in the other. As there was no *Heterakis* material on hand, both groups of turkeys were fed a dry mash to which dirt from a hen yard had been added in the proportion of eight parts of mash to

[2] Tyzzer, E. E., Fabyan, M., and Foot, N. C., *J. Infect. Dis.*, 1921, xxix, 268.

[3] Wegeforth, H. M., and Wegeforth, P., *J. Pharmacol. and Exp. Therap.*, 1921, xvii, 249.

one of dirt. A shovelful of the same dirt was thrown into the cages from time to time.

The two groups of turkeys were treated similarly in every respect except that throughout the experiment ipecac was added to the food of the eleven confined in one of the cages, the twelve in the other cage serving as controls. In order that the effect of ipecac on inoculated blackhead might also be tested, one of the controls and one of the ipecac-treated turkeys had been inoculated on August 10. Powdered ipecac was employed and for the first 6 days was given in a dry mash, so that the turkeys, averaging at first 300 gm. in weight, would each get about 0.08 gm. per day. Since the mash was not all eaten and became somewhat scattered, the amount of ipecac actually taken was probably considerably less than the dose furnished.

Beginning on August 17 ipecac was added to the daily ration of sour milk in an amount equivalent to 0.1 gm. for each turkey. The average body weight was now 400 gm. Although the drugged milk was taken slowly it was all cleaned up each day so that the dosage of ipecac was now between 0.1 and 0.18 gm. On August 19 ipecac was added to the milk in an amount equivalent to 0.2 gm. per turkey and was no longer furnished in the mash. On August 29 the dose was doubled, but as the turkeys then refused the milk, it was necessary on the following day to return to the previous dose of 0.2 gm. Although the birds had by this time greatly increased in weight, several were already showing symptoms of blackhead.

The detailed results of these various procedures are given in Table II.

The results obtained from the two inoculated turkeys show that ipecac has no specific action on the organisms of blackhead, as it occurs in the tissues of the turkey, the course of the disease being the same in both control and treated birds.

It is evident from this experiment that ipecac administered as a prophylactic in large doses has a distinct effect in that treated turkeys remain healthy longer than untreated birds when subjected to conditions such as might be furnished by the ordinary farmyard. Thus 63 per cent of the controls had died before any death had occurred among the treated turkeys, and 70 per cent of the latter were alive at the death of the last of the control turkeys.

Since this retarding effect of ipecac on the incidence of the natural type of infection occurred, notwithstanding the absence of any effect on the inoculated disease, it seemed important to ascertain whether the ipecac treatment might not tend to affect the development of *Heterakis*. These worms were found, however, to be as numerous in the treated as in the control turkeys. The inoculated turkey

TABLE II.

Group.	Turkey No.*	Treatment.	Death.	Anatomical findings.	*Heterakis.*
			1921		
	42†	From Aug. 11, 1921, dirt from hen yard in food and in cage.	Aug. 27. D.	Inoculated blackhead together with typical lesions of cecum and liver.	300 (estimated)
1	43		Sept. 1. "	Typical blackhead.	+
Controls.	44		" 8. "	" "	+
	45		" 3. "	" "	165
	46		Aug. 23. "	" "	81
	47		" 28. "	" "	+
	48		" 28. "	" "	+
	49		Sept. 1. "	" "	+
	50		Aug. 31 "	" "	+
	51		Sept. 3. "	" "	+++
	52		" 4. "	" "	+++
	53		Aug. 28. "	" "	++
	54†‡	From Aug. 11, 1921, dirt from hen yard in food and cage. From Aug. 11, 1921, powdered ipecac at first in doses of 0.08 gm., gradually increased to 0.2 gm.	Aug. 28. D.	Inoculated blackhead; ceca normal.	356
	55		Sept. 23. "	Typical blackhead.	27
2					
Ipecac-	56		" 3. "	" "	293+
treated.	57		Alive.	(Symptoms of blackhead; recovery.)	
	58		Sept. 12. D.	Typical blackhead.	+
	59		Oct. 6. "	" "	92
	60		Sept. 11. "	" "	+
	61		" 4. "	" "	+
	62		" 26. "	" "	150+
	63‡		" 2. "	" "	59
	64‡		" 10. "	" "	+

* Turkeys hatched July 14, 1921.

† Aug. 10. Breast inoculated with liver lesions of Turkey C. P. S. 21.65.

‡ Previously treated with wine of ipecac.

(No. 54) which received ipecac treatment, and in which the ceca were not infected, nevertheless harbored a large number of worms.

In the absence of any specific action of the drug either on the organism of blackhead in the tissues or on the intestinal worm, *Heterakis papillosa*, explanation was now sought in some simple physiological action of the drug such as the increase of secretions or peristalsis. On one occasion it was thought that the droppings of the treated turkeys were more watery than those of the controls, but continued observations showed no marked difference in this respect between the two lots. The administration of ipecac in the food had, under the conditions of the experiment, a distinct retarding effect on the growth of the turkeys, possibly because a smaller amount of the drugged food was taken. No other untoward effect was noted unless the appearance of abnormalities affecting both bones and feathers in the only turkey which recovered is to be attributed to the long course of treatment.

Sulfur Treatment.—From the results obtained with ipecac it was thought probable that other drugs having evacuant properties would have a similar action. Sulfur was chosen because it is non-toxic, can thus be administered in large amounts, and is said to increase peristalsis. The number of turkeys available at this time was small.

Experiment 5.—Four turkeys of the lot of forty hatched on July 14, 1921, and five hatched on August 14 were employed. On September 1 these turkeys, now 48 and 17 days old respectively, were distributed in four cages so that some of either age could be furnished sulfur daily in their food, while the others would serve as controls. From the beginning of the experiment dirt from a hen yard was scattered in all the cages and used to contaminate the food. The younger of the turkeys treated each received one teaspoonful of precipitated sulfur twice a day, one-half being added to the sour milk, one-half to the dry mash, while the older ones each received two teaspoonfuls twice a day, similarly added to the dry mash and the sour milk. Later on the two groups that were being fed sulfur were combined in one cage and the controls in another. It was now no longer possible to estimate the dosage for each turkey but an abundance of sulfur was always available in both milk and dry mash. Sulfur, since it is a practically tasteless powder, is readily taken with the dry mash but tends to float on a fluid like milk.

The results obtained are given in Table III.

It is quite apparent that sulfur may be administered in large amounts to turkeys without untoward effect. The droppings, es-

pecially those of the cylindrical type from the small intestine, may assume a dull clayey appearance. No diarrhea was noted.

Although the number of turkeys employed was small, the results are suggestive in that with one exception the treated turkeys survived longer than the controls. It was necessary to kill two of the smaller turkeys (one control and one sulfur-treated turkey) early in the ex-

TABLE III.

Group.	Turkey No.	Treatment.	Death.	Anatomical findings.	*Heterakis*.
			1921		
	67*	From Sept. 1,	Sept. 29. D.	Typical blackhead.	47
	68*	1921, dirt from	" 26. "	" "	350 (esti-
1	71†	hen yard placed	" 20. K.	(Sept. 15. Opera-	mated).
Controls.		in cage and also	(weak legs).	tion on crop.)	
		in food.		Typical blackhead.	45+
	72†		Sept. 24. D.	" "	6
					(1 macer-
					ated).
	69*	From Sept. 1, 1921, dirt from hen yard placed in cage and also in food. Sulfur in daily ration.	Sept. 25. D.	Typical blackhead. (Sept. 9. Operation on crop.)	28
2 Sulfur-treated.	70*		Oct. 11. "	Typical blackhead.	13+
	73†		Sept. 20. K. (weak legs).	" "	14+
	74†		Oct. 3. K.	" "	9
	75†		" 3. D.	" "	16

* Hatched July 14, 1921.

† Hatched Aug. 14, 1921.

periment (September 20), on account of helplessness resulting from faulty development. Both of these showed lesions in ceca and liver.

In general, *Heterakis* was found in the control turkeys in greater numbers than in the sulfur-fed turkeys. In the control turkey showing only six worms, the finding of a barely recognizable macerated *Heterakis* in the putrefying material from the occluded cecum suggests that worms are destroyed in the decomposition that takes place when these organs become extensively diseased.

DISCUSSION.

Since Graybill and Smith[4] have called attention to *Heterakis papillosa* as a possible factor in the transmission of blackhead, it appears to be a matter of primary importance to establish the part played by this worm in the natural incidence of this disease. In a previous paper[2] statistical data were discussed, showing that no great preponderance of *Heterakis* had been found in infected as compared with normal turkeys. The opinion was also expressed that invasion of the mucosa might be effected by pathological conditions arising from other causes as well as from the presence of *Heterakis*. Reliance on the post-mortem count of *Heterakis* may, however, not be justified in all cases, for it is probable that many worms are destroyed when the cecum becomes extensively diseased, as already mentioned. It is also notable that in cases in which only one cecum is diseased, there are usually fewer worms in the abnormal than in the normal cecum.

The susceptibility of the turkey, however, is evidently so great that infection may occur with very few and possibly with no *Heterakis* present. A half grown turkey recently killed showed characteristic blackhead with involvement of one cecum and the liver. The diseased cecum showed a localized lesion not over 2 cm. in extent, while the other appeared quite normal. Only one immature *Heterakis* was found on careful search of both ceca.

Evidence of the occurrence of pathological conditions in the ceca independently of *Heterakis* and unassociated with blackhead infection has already been pointed out in the cores which sometimes form in these organs at an early age without any blackhead infection. Such cores were recently found in the ceca of two turkeys 5 and 8 days old, respectively, that had been kept in a clean compartment and fed sterilized food. In these also there was no evidence of blackhead.

Finally, our previous suggestion that blackhead may occur spontaneously in the absence of *Heterakis* is to a certain extent supported by the experimental production of the common type of the disease by feeding liver lesions from acute cases. Not only was *Heterakis* presumably absent from the material fed, but it could not be demonstrated post mortem. Young turkeys under natural conditions, however, would not have access to the virus in tissues.

[4] Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, xxxi, 647.

As to the sources of the virus in the natural transmission of blackhead, we have now experimental evidence suggesting that it may be present in the eggs of *Heterakis*, although this has not as yet been confirmed by morphological evidence. In the present investigation it was found possible to produce blackhead by feeding ripe *Heterakis* ova that had been subjected to 1.5 per cent nitric acid sufficiently long (3 days) to render the material bacteriologically sterile. It is hardly probable that the virus would survive under such conditions unless protected by the impervious membranes of the *Heterakis* ovum, for all our studies indicate that *Histomonas* is a relatively non-resistant organism. The second possibility is that the virus is present in the turkey from the time of hatching, awaiting only some exciting factor to cause it to invade the tissue. The microscopic study of tissues of many young turkeys has never revealed *Histomonas* except in blackhead infection, in which it is rarely found outside the living tissues. Furthermore, all isolated control turkeys, even those showing evidence of pathological conditions, *i.e.* cecal cores, failed to develop blackhead.

That it is only necessary to feed acid-treated *Heterakis* ova to young turkeys kept in strict isolation to produce the disease suggests that the virus is transmitted from bird to bird in the ovum of *Heterakis*. Although infection was obtained in 100 per cent of the ova-fed turkeys in certain of the experiments already outlined, postmortem examination of a large number of turkeys has already shown that the introduction of a small number of the worms may not always produce the disease. Again the disease may occur in association with very few worms. Such irregularity with respect to infection might be accounted for by the absence of *Histomonas* from a certain proportion of *Heterakis* eggs but, without morphological demonstration, this assumption is unwarranted.

There is considerable variation in the interval between the ingestion of ova and the onset of the disease. Whether this is due to the absence of *Histomonas* for a time or to conditions that prevent immediate invasion of the tissues is not known. It is also wholly problematical whether this parasite is able to persist and multiply in the cecal contents before invading the tissues. If *Histomonas* were actually introduced into the cecal mucosa by the worm larvæ, it would be difficult to account for such irregularity in the onset of infection as may, for

example, be seen in Experiment 3. In this experiment, although a number of the turkeys when killed relatively early (after 8 to 11 days) showed numerous larvæ, only one was diseased and in this case the infection was quite advanced. The efficacy of this *Heterakis* material was proved by the fact that two other turkeys fed with a portion which had also been treated with dilute nitric acid developed the disease. As no symptoms appeared in these turkeys for a period of 3 weeks, it is probable that they did not become immediately infected.

Graybill and Smith[4] have considered *Heterakis papillosa* as a preliminary agent in that, by injury to the cecal mucosa, it may serve to lower the resistance of the turkey preparatory to the invasion of the protozoon. While this might not appear improbable, the presence of the protozoon in the ceca prior to the ingestion of *Heterakis* ova has not been demonstrated. Furthermore, little is known as to the invasive ability of *Histomonas*, although we have shown that the injection of material containing large numbers of this organism directly into the lumen of the cecum may fail to produce infection. While it is possible that the degree of the injury and the extent of the infection may depend on the dosage of *Heterakis* ova, it is known that blackhead may in some instances follow the ingestion of only a small number of these eggs. We have also found that infection may occasionally be produced, in the absence of *Heterakis* and its injurious effects, by merely feeding liver lesions containing large numbers of the protozoon. That injury of the cecal mucosa will of itself lead to blackhead infection is not borne out by the facts. Cores associated with inflammation in the ceca of young turkeys kept isolated and on a diet of sterilized food furnish an example of injury to these organs in the absence of blackhead infection. The results of the present experiments would indicate that *Histomonas* is not present in newly hatched turkeys but is acquired naturally by them as the result of the ingestion from contaminated soil of ripe *Heterakis* ova containing this protozoon. It may also be introduced experimentally by feeding fresh liver lesions. *Heterakis*, aside from any injurious effect that it may have on the cecal mucosa, would in this case be an important distributing agent of the protozoon of blackhead. However, the microscopic examination of large numbers of *Heterakis* eggs at all stages of development has thus far failed to reveal any inclusion resembling *Histomonas*.

The administration of drugs for the prevention or cure of blackhead has not been successful as employed in the present experiments. The attempt was made to obtain the maximum effect of the drugs by adding large doses to the daily food ration.

Powdered ipecac appears to have no deleterious effect on the blackhead organism in the tissues for it does not prevent the development of blackhead resulting from inoculation nor does it prevent the fatal termination of this form of the disease. One would not therefore expect it to have curative properties when used in natural blackhead. When administered prophylactically it has a definite effect in delaying the onset of the disease. This effect is not the result of any injurious action on *Heterakis*, for this worm was found in great numbers in the ipecac-treated turkeys. Ipecac served only temporarily to prevent blackhead infection, in that the treated turkeys eventually became infected and only one of the ten recovered. The claims of Wegeforth[3] with respect to both the prophylactic and curative properties of ipecac are thus disproved by the present investigation. Since this drug showed no evident effect on either blackhead virus or on *Heterakis*, it was thought that some physiological action such as the stimulation of secretions and peristalsis might account for its tendency to delay invasion. Accordingly sulfur, on account of its mild evacuant properties, was administered to turkeys which were at the same time exposed to infection.

Sulfur in large doses appears also to retard infection, although the number of turkeys employed was not sufficiently large to furnish conclusive results. Sulfur may be slightly unfavorable to *Heterakis*, for in general the sulfur-treated turkeys showed fewer of these worms than the control birds.

The failure of *Chaparro amargosa*, an efficient remedy for amebic dysentery in the human being, to prevent blackhead in turkeys has been reported in a previous paper.[5] Since the blackhead parasite proves to be of the nature of a flagellate rather than an ameba, it is not remarkable that it does not respond to drugs having amebicidal properties. However, the failure of tartar emetic and of quinine to alter the course of blackhead is no less marked,[6] although the value of these drugs in certain protozoan infections is well established.

[5] Tyzzer, E. E., *J. Med. Research*, 1919–20, xli, 211.

[6] Tyzzer, E. E., and Fabyan, M., *J. Infect. Dis.*, 1920, xxvii, 207.

At present the most hopeful mode of attacking blackhead appears to be through measures tending to eliminate *Heterakis*. Such measures may be directed towards the isolation of the turkey or towards the destruction of the worm. It is possible that vermifuges administered at short intervals may prevent blackhead infection. However, it is not, at present, known how soon after the introduction of *Heterakis* the tissues are invaded by the blackhead parasite. Invasion may occur very early in some cases, for extensive infiltration of the tissues has been found in a very young turkey 8 days after feeding *Heterakis* material. It is safe to assume that infection occurred in this case at least as early as the 4th or 5th day. The interval between the ingestion of *Heterakis* ova and the invasion of the tissues by the parasite of blackhead may therefore be very brief, in fact it is conceivable that infection may follow immediately in some cases. Whether the administration of vermifuges at intervals will either prevent or lessen the incidence of blackhead in young turkeys must be determined by further experimentation. For the present it appears to be the safer course to rely on isolation for the prevention of the introduction of *Heterakis* to young turkeys.

The successful employment in the present investigation of soil from a hen yard for the purpose of producing blackhead furnishes additional experimental evidence as to a common source of this infection. The agencies here used for the experimental production of blackhead are present and operative in most farmyards. While it is already well established that blackhead occurs in chickens, little is known concerning its prevalence among them. During the present season typical blackhead lesions of the liver as well as of the ceca have been found in young chickens raised on ground previously occupied by mature poultry. It is probable that blackhead tends to run a mild course in young chickens, as already indicated by the experiments of Smith and Graybill,[7] and on this account is often not recognized. That the disease of the chicken and the turkey are identical, as indicated by the transmission of blackhead to turkeys by *Heterakis* material obtained from hens, is further shown by the production of typical blackhead in the turkey through the inoculation of the latter with the liver lesions from a case of blackhead in a chicken.

[7] Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1920, xxxii, 143.

Blackhead was inoculated into nine turkeys during the course of the present season and three of these that developed active lesions completely recovered. Subsequent repeated subcutaneous inoculations of active virus were without effect. That this immunity may, however, be of short duration is indicated by the fact that, kept on infested ground, one of the above turkeys apparently passed through a mild attack of blackhead about 3 months later. We have already reported the occurrence of the disease in older breeding turkeys, male and female, and also in a hen over 2 years old. Recently an acute process was found in the cecum of a 3 year old male turkey which had always been confined with other turkeys in a small yard, where every external condition necessary for infection seemed to be present. It is evident that turkeys may have attacks of more or less severity from time to time. Occasional cases of the disease are also found in old fowls that had presumably been exposed to the infection throughout life.

The experiments conducted in the present investigation have again shown that newly hatched turkeys do well in close confinement, and notwithstanding the sterilization of the food they may develop normally for at least a month. Young turkeys infected with blackhead evidently resist the disease longer when confined than when wandering free, possibly through the elimination of the fatigue factor.

Previous conclusions as to the flagellate nature of *Histomonas meleagridis* are confirmed by observations made in the course of the present investigation. By the strict isolation of turkeys and by furnishing them sterilized food, it has been possible to keep them free from all protozoan contamination for a period of 4 weeks. Blackhead has been produced in such turkeys by feeding them *Heterakis* ova that had been treated in dilute nitric acid with the object of destroying all organisms external to the egg membrane. Under such conditions *Histomonas* was found in flagellate form in the ceca late in the disease. The flagellum of this organism was also observed in motion in incubated material from acute liver lesions. The morphological details of the flagellated forms will be considered in a subsequent paper.

CONCLUSIONS.

That the ovum of *Heterakis papillosa* is an important source of the virus in the natural transmission of blackhead in turkeys is suggested by experimental evidence obtained in the present investigation. Confirmatory morphological evidence has not thus far been obtained to establish definitely that this worm serves as the invertebrate host of *Histomonas meleagridis*.

It is possible occasionally to produce blackhead in turkeys by feeding large amounts of the virus alone, as obtained in liver lesions. This artificial procedure is, however, not duplicated in nature since the lesions of the disease are not eaten by young turkeys.

The intentional contamination of the food of young turkeys with dirt taken from hen yards has in the present experiments invariably produced blackhead. The conditions provided are essentially those that prevail in many farmyards.

The identity of blackhead infection in common fowls with that of turkeys is indicated by the production of the typical disease in a turkey by the inoculation of liver lesions obtained from a diseased chicken.

It is not known whether variation in the period between the ingestion of *Heterakis* ova and the onset of the disease is dependent on the absence of the virus for a time or on the absence of the conditions necessary for invasion.

Under experimental conditions a large proportion of the *Heterakis* ova fail, at least when fed by hand, to hatch out in the intestine, but, passing through, may later be taken up with contaminated food. This is shown by the occurrence in the ceca of larvæ in various stages of development long after the *Heterakis* material is fed.

Ipecac, like other drugs previously tested, is found to be of no practical value in the control of blackhead. It has no deleterious effect on *Histomonas* in the tissues and does not prevent the development of *Heterakis*. Its daily administration to the limit of tolerance fails to prevent infection or a fatal outcome in the naturally acquired disease.

The fact that ipecac may delay the onset of the infection may perhaps be due to some physiological property of this drug. Sulfur administered daily in large amount likewise, possibly on account of its evacuant action, tends to delay infection.

Recovery from inoculated blackhead is associated with a degree of immunity. That this is not permanent is indicated by the appearance of symptoms of blackhead several months after recovery from the inoculated disease. The fact that blackhead sometimes appears in mature turkeys that have been kept constantly on infested ground suggests that these birds may suffer from repeated attacks.

Observations made during the course of the present investigations confirm previous conclusions as to the flagellate character of *Histomonas meleagridis*.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VIII. Further Observations on the Cultural and Morphological Characters of Bacterium pneumosintes.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 67.

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In an earlier paper of this series[1] *Bacterium pneumosintes*, derived from the nasopharyngeal washings of patients in the early hours of acute epidemic influenza, was described as a minute bacilloid body of regular form, with a length about two to three times its breadth, measuring 0.15 to 0.3 micron in the long axis. It was stated that although longer individuals were seen occasionally, "the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape."

At that time, *Bacterium pneumosintes* had been cultivated only in a tissue medium composed of human ascitic fluid and fresh rabbit kidney, sometimes with the addition of beef infusion broth and nutrient agar. The combination of ascitic fluid and fresh kidney tissue has remained the medium of choice for the maintenance of the cultures, because in it they require transfer only at long intervals and they retain their morphological and cultural characteristics through many generations. But during cultivation over a period of 1 to 3 years the three strains[2] at present available have become saprophytic, so that at present they are cultivable anaerobically in a variety of media which are less difficult to prepare. Coincident with this adaptation to a new environment, certain variations in morphology and a loss of pathogenicity for rabbits have been observed. It is the

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

[2] These strains were derived from Cases 16, 17, and 26 (Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125).

purpose of this paper to describe the cultural and morphological characteristics of *Bacterium pneumosintes* after prolonged artificial cultivation. The three strains have behaved in an identical manner, and a common description will suffice.

Methods of Cultivation.

Even after cultivation for 3 years *in vitro Bacterium pneumosintes* fails to grow in autoclaved media without the nutritive or growth-stimulating factors found in fresh tissue or body fluids. But the ascitic fluid of the original medium may be replaced by peptone broth; and fresh defibrinated blood, vegetable tissue,[3] or the products of bacterial metabolism may be substituted for rabbit kidney. The addition of dextrose (1 per cent) to the medium hastens the establishment of anaerobic conditions, and, judging from the rapidity and density of the growth, increases its nutrient value.

The basis of the media now in use is a 1 per cent peptone beef infusion broth, with sodium chloride 0.5 per cent, titrated to pH = 7.4 on the Sörensen scale. According to the object for which the culture is prepared, this broth is enriched with fresh blood, animal or vegetable tissue, or by the growth of other organisms, as will be described. Solid and semisolid media are made by the addition of nutrient agar in the proper proportion.

When a large inoculum from an enriched medium is added to dextrose broth alone, *Bacterium pneumosintes* grows in the primary, but not in subsequent transplants, showing that the initial growth is dependent upon factors carried over from the tissue medium. Thjötta and Avery[4] have shown how minute is the quantity of the growth-stimulating factors required in the case of Pfeiffer's bacillus. Their V factor in dilutions of 10^{-3} and their X factor in dilutions of 10^{-6} suffice to promote growth. With *Bacterium pneumosintes* the

[3] Dr. O. T. Avery and Dr. H. J. Morgan, at the Hospital of The Rockefeller Institute, found that *Bacterium pneumosintes* grows readily in a simple infusion broth containing a fragment of fresh vegetable tissue (potato, turnip, parsnip, etc.) and kindly permit us to report their observation in advance of their own publication. They described this medium in the *Proceedings of the Society for Experimental Biology and Medicine*, 1921, xix, 113.

[4] Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.

essential growth-stimulating factors appear to be of the same order of efficacy. For example, 20 mg. of fresh rabbit kidney supports growth in 5 cc. of medium. The faint haze of *Bacillus coli* which develops in the 1st hour after inoculation makes dextrose broth a highly favorable medium for *Bacterium pneumosintes*. This interesting symbiotic relationship will be discussed in a subsequent section.

Cultural Characteristics.

Bacterium pneumosintes was described as an obligate anaerobe and has maintained that character. We have successfully cultivated the organism only under a vaseline seal or in the depths of the medium in the presence of active reducing agents such as dextrose, fresh animal or vegetable tissue, or aerobic organisms, or in a strictly anaerobic jar. Under such conditions, under a vaseline seal for example, *Bacterium pneumosintes* grows in enriched dextrose broth in a diffuse cloud throughout the fluid medium. In previously incubated tubes, in which anaerobic conditions are already established, this cloud becomes visible within a few (8 to 16) hours after inoculation and reaches its greatest density in 3 to 5 days, when the culture is opaque by reflected light and shows a smoky translucency by transmitted light. Growth is then checked by acid production. The cloud is too finely divided to show a bacterial shimmer, and remains in suspension for days, gradually settling in an even, amorphous, cream-colored layer in the bottom of the tube. Spontaneous flocculation has not been observed.

In the depths of solid media submicroscopic colonies develop as tiny gray specks, which, under the high power of the microscope, are found to be dense irregular masses of bacteria with a fringe of single organisms.

Dextrose is split by *Bacterium pneumosintes* with acid formation but without gas production. In dextrose broth the limiting hydrogen ion concentration is the same for all three strains; namely, 5.2 to 5.3 on the Sörensen scale. After growth has apparently ceased, however, the organisms remain viable for several days in the acid medium.

Recently the simple, safe, and efficient anaerobic jar described by Brown[5] has furnished us with the conditions necessary for the

[5] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

development of surface colonies of *Bacterium pneumosintes*. The jar is a modification of those devised by McIntosh and Fildes[6] and by Smillie,[7] and utilizes the catalytic activity of palladinized asbestos heated by a resistance coil in a wire-screened chamber (the principle of the Davy safety lamp) for the union of the oxygen with hydrogen. In this jar we have used plates and slants of nutrient agar, made with beef infusion broth without dextrose, enriched with 5 per cent of fresh defibrinated rabbit blood.

After incubation for 7 to 10 days blood agar plates sown with *Bacterium pneumosintes* show many very minute colonies, almost submicroscopic in size, which are round, raised, and convex, with an entire edge and a colorless translucency. No characteristic structure has been observed, and the growth does not discolor or precipitate the medium. On account of their minute size the colonies are usually discrete, even when close together, but they coalesce to form raised plaques of confluent growth in the most crowded areas.

These colonies interest us as the first example of which we are aware of surface colony formation by a filter-passing obligate anaerobe. The plates also give us a ready means of purifying contaminated cultures, and are useful for the demonstration of organisms in sparse growths of early generations in the ascitic fluid-kidney medium in which the microscopic observation of *Bacterium pneumosintes* is difficult on account of its minute size and the presence of stained protein precipitate.

Morphology.

The substitution of dextrose-peptone broth for ascitic fluid or serum in the medium results in a considerable change in the morphology of *Bacterium pneumosintes*. The dextrose, by the prompt establishment of anaerobic conditions and by its nutritive value to the organisms, supports a much more luxuriant growth than is found in ascitic fluid media. The bacteria are found in diplo form, or in chains of several members, and many of the individual organisms have increased in length so as to be obviously bacillary. They are plump rods with rather pointed ends, which give them a spindle

[6] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.
[7] Smillie, W. G., *J. Exp. Med.*, 1917, xxvi, 59.

shape. Stains color them deeply only in the middle and fade out towards the ends. In chains the spaces between the members are sharply demarcated. More variation occurs in length than in thickness. Forms from 0.5 to 1.0 micron long are not uncommon in cultures which show the characteristic minute forms also. Figs. 1 and 2 show the relative size and shape of *Bacterium pneumosintes* in a collodion sac dialysate of ascitic fluid and in dextrose broth respectively. A strain which has grown as fusiform bacilli in dextrose broth for several generations reverts to the original minute form on cultivation in a dialysate of the original ascitic fluid medium (Fig. 3). Aside from the differences in length and the greater tendency to chain formation, no irregularities in morphology have been noted in comparison with the early generations.

In films from surface colonies grown for 7 days on rabbit blood agar in an anaerobic jar, most of the organisms are of the minute form, but here also longer individuals are found.

As stated previously, *Bacterium pneumosintes* decolorizes by Gram's method. The organisms stain with the usual basic dyes. They are not motile. Neither capsules nor flagella have been demonstrated.

Serological Reactions.

The fusiform, bacillary forms of *Bacterium pneumosintes* grown anaerobically in enriched dextrose broth media and on blood agar plates have been tested at intervals with the immune rabbit sera produced a year ago with cultures of the organism grown in the collodion sac dialysate of an ascitic fluid-rabbit kidney medium.[8] All three strains are promptly agglutinated by these sera, and, on the other hand, they show no tendency to spontaneous flocculation or to agglutination in normal serum. Their genetic relationship to the original minute forms of *Bacterium pneumosintes* is without question, and is further evidenced by their strictly anaerobic character and their reversion to the minute forms on transfer to the original medium.

Symbiosis.

Mention has already been made of the symbiotic development of *Bacterium pneumosintes* with *Bacillus pfeifferi*, the pneumococcus,

[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

Streptococcus hæmolyticus and *viridans*, and staphylococci, in cultures accidentally contaminated with these organisms.[1] In a number of instances *Bacterium pneumosintes* was recovered from these mixed cultures by filtration. Since the medium then in use was sufficient for the independent growth of any of these organisms, the coincident development of *Bacterium pneumosintes* and the contaminating organism did not give evidence of a nutritive interchange between them.

More recently, in cultures intentionally inoculated with a strain of *Bacillus mesentericus*, we have observed the growth of *Bacterium pneumosintes* in autoclaved dextrose broth in the absence of a fresh tissue fragment. This observation has led to the development of a simple method of anaerobic cultivation. Aerated dextrose broth is inoculated with a young culture of *Bacillus mesentericus* in which spores have not yet formed. During the first hours after inoculation the organism grows diffusely in a faint cloud without pellicle formation. The reducing activity of this obligate aerobe is shown by the decolorization of methylene blue in such a culture within a few minutes after its introduction. The culture is sealed with vaseline when inoculated, or shortly afterward and growth is inhibited by the exhaustion of free oxygen from the medium. Meanwhile the hydrogen ion concentration of the medium falls from 7.4 to 7.2–6.8. 6.8 appears to be the limiting acidity for our strain of *Bacillus mesentericus*. If such a preparation is then inoculated with *Bacterium pneumosintes*, the anaerobe grows luxuriantly and clouds the medium heavily in the course of 1 or 2 days.

This rapid growth of *Bacterium pneumosintes* carries the hydrogen ion concentration of the medium to 5.3–5.2 in the course of 3 or 4 days. Coincidently the vegetative forms of *Bacillus mesentericus* are killed and undergo autolysis so that finally microscopic films and plate cultures may show only the anaerobic organisms. Usually, however, some few spores of *Bacillus mesentericus* are introduced in the original inoculum or are formed in the culture. These may develop subsequently and complicate results. We have therefore tested a number of other organisms for their nutritive or growth-promoting properties, combined with other characters which make them suitable for use. Of these, *Bacillus coli communis* seems well adapted to our purpose. It multiplies rapidly in plain or dextrose

broth under a vaseline seal, so that a sufficient growth is obtained within an hour or two after inoculation. During this short incubation the hydrogen ion concentration of the medium is only slightly affected. The organism may then be killed by exposure to 100°C. for 15 to 30 minutes without destroying the peculiar nutritive substances it imparts to the medium. At the same time anaerobic conditions are immediately established under the vaseline seal. *Bacillus coli* is practically non-pathogenic, and its autolyzed products are not toxic in the minute amounts contained in these cultures. The *Bacillus coli* broth is inexpensive and simple to prepare, and less liable to contamination than are unheated media containing fresh tissue or blood. It contains a minimum of foreign protein. Finally, it supports an abundant growth of *Bacterium pneumosintes* in successive generations. A large inoculum, 0.2 to 0.5 cc. of fluid culture, should be used for seeding.

Preservation of Stock Cultures.

For the maintenance of our stock cultures we have heretofore relied upon the original ascitic fluid-tissue medium, in which *Bacterium pneumosintes* after a preliminary incubation of 5 to 7 days has remained viable at room temperature for $2\frac{1}{2}$ years. Recently blood broth, without dextrose, has been used for the same purpose with encouraging results. The tests are incomplete and the limit of viability in this medium is not yet known, but *Bacterium pneumosintes* established in artificial culture appears to be a resistant organism, and probably requires transfer only at long intervals. We have also employed the method described by Swift[9] for the preservation of microorganisms by freezing and drying *in vacuo*. *Bacterium pneumosintes* withstands this process. The dried cultures are viable for at least 2 months.

In conclusion, we have at present available a number of cultural methods and culture media suitable for a variety of purposes.

For primary isolations from filtered nasopharyngeal secretions, or from the lung tissues of affected animals, the Smith-Noguchi ascitic fluid-rabbit kidney medium probably insures success in the largest percentage of cases. It was in this medium that the original

[9] Swift, H. F., *J. Exp. Med.*, 1921, xxxiii, 69.

isolations were made and the morphological and cultural characteristics of *Bacterium pneumosintes* were first observed.

For the maintenance of stock cultures also, the ascitic fluid-fresh tissue medium is preferred. Blood broth probably may be substituted in the case of well established cultures. *Bacterium pneumosintes* withstands freezing and drying *in vacuo* and probably is viable for long periods in the dry state.

For the demonstration of sparse growths of the microorganisms in the tissue medium, blood agar plates in an anaerobic jar have advantages over the microscope. They are useful also for the purification of contaminated cultures.

Finally, for immunization and for serological reactions requiring considerable quantities of bacteria with a minimal amount of foreign protein, dextrose broth cultures, enriched by the growth of another microorganism, furnish the most useful product. Suitable suspensions may be obtained also from blood agar plates or from growths in the dialysate liquid of collodion sacs containing the culture medium.

These methods of cultivation are being used in studies of the nasopharyngeal secretions of influenza patients obtained during the present recurrence of influenza in New York City. The results of these studies will be reported in subsequent papers of this series.

SUMMARY.

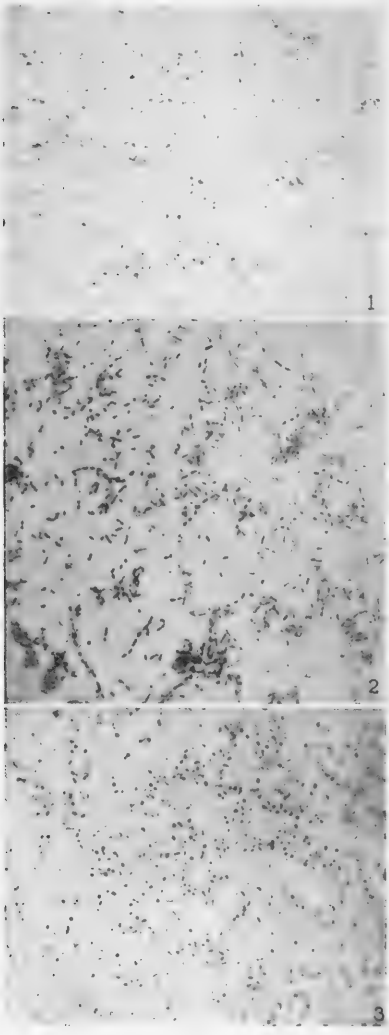
After artificial cultivation for a period of over 3 years *Bacterium pneumosintes* has maintained its original morphological and cultural characteristics, when grown in the original medium. Adaptation to a saprophytic existence has been accompanied by a loss of pathogenicity. Our strains now grow readily under strictly anaerobic conditions in a variety of media with peptone broth as a base, enriched with fresh tissue, blood, or by the growth of other bacteria. Surface colonies have been obtained on blood agar plates in an anaerobic jar. These various methods of cultivation are adapted to special purposes. In broth cultures *Bacterium pneumosintes* grows in larger forms than in the ascitic fluid-tissue medium, but the identity of the microorganisms is proved by their serological reactions and by reversion to the minute forms on transfer to the original medium.

EXPLANATION OF PLATE 67.

FIG. 1. *Bacterium pneumosintes*, Strain 16, in the twenty-fourth generation. Originally isolated March 30, 1919. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium. × 1,000.

FIG. 2. *Bacterium pneumosintes*, same strain. Film from a culture in dextrose broth and rabbit kidney medium. × 1,000.

FIG. 3. *Bacterium pneumosintes*, same strain. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium seeded from a culture in dextrose broth and rabbit kidney. Note the reversion to the original form. × 1,000.



(Olitsky and Gates: Nasopharyngeal secretions from influenza. VIII.)

THE PEPTASE, LIPASE, AND INVERTASE OF HEMOLYTIC STREPTOCOCCUS.

By FRANKLIN A. STEVENS,* M.D., and RANDOLPH WEST, M.D.

(*From the Department of Medicine of the College of Physicians and Surgeons of Columbia University and the Presbyterian Hospital, New York.*)

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The study of the metabolic functions of cells and of more highly organized forms of life has shown that in many instances enzymes are responsible for the cleavage of complex molecules into their simpler components which may be utilized to support life and growth. While many of these enzymes are found in groups of plants or animals in which they accomplish the conversion of certain substances peculiar to individual species, others are more widely distributed. Among the latter group are those necessary for the disintegration of carbohydrates, fats, and proteins into the elementary molecules which are assimilated by the individual cell, so that it is not surprising that they have been found in bacterial extracts, because most bacteria are able to utilize complex organic materials for food.

It has been shown that all bacteria do not possess enzymes for the digestion of natural protein and that variations may be found in the heat resistance and action of peptolytic and sugar-splitting enzymes obtained from different bacteria. On account of these apparent differences in the identity of bacterial enzymes, the peptase, lipase, and invertase of the hemolytic streptococcus have been studied so that they may be compared with similar enzymes of other bacteria. It was hoped that further application of the methods employed would reveal differences sufficient to account for the variations in the pathogenicity of different strains of this streptococcus.

Methods.

Preparation of the Solution of Enzyme.—The enzyme solutions used in the following experiments were prepared from broth cultures

* Coolidge Fellow, Columbia University.

of a beta type hemolytic streptococcus. This strain was originally obtained from a fatal case of peritonitis. Since the objects of the experiments were to perfect a method by which active solutions could be obtained and to study the physical conditions optimum for the action of these solutions on various substrates, no other strains were studied. Active hemolysis could be obtained with this streptococcus on blood agar plates and the centrifugates or filtrates of actively growing broth cultures were hemolytic. The strain fermented lactose and salicin but did not produce acid in mannitol broth. The final hydrogen ion concentration of cultures in 1 per cent dextrose broth was pH 5.0.

The streptococci from which the enzymes were extracted were obtained by the sedimentation of broth cultures after 12 to 16 hours of active growth. The broth was prepared in the usual way except that it contained 2 per cent peptone instead of the 1 per cent solution ordinarily employed in bacteriologic work. Since the tap water used in the broth contained considerable inorganic material, the first lots of media prepared precipitated heavily during sterilization. It was impossible to obtain clean bacteria when this precipitation occurred. While the broth could be filtered and autoclaved a second time, bouillon which was first titrated to pH 0.9 and boiled a few minutes at this H ion concentration, then filtered and titrated to pH 8.0 gave no further precipitate. The media prepared in this way yielded a greater quantity of bacteria than lots which were twice autoclaved. The strain employed agglutinated spontaneously in this medium so that the streptococci settled to the bottom of the flask; the supernatant fluid could then be decanted, leaving the bacterial sediment in the remaining few hundred cubic centimeters. Due to the incomplete sedimentation and the scant yield from large flasks of media, 0.1 per cent dextrose was added before sterilization. Large quantities of bacteria could then be obtained within a few hours after the flasks were seeded; the final acid concentration was between pH 6.0 and 6.5 in the dextrose broth cultures. Dextrose was used in the preparation of all eyzyme solutions except in a few experiments designed to determine the effect of acid on the enzymes. It increased the growth and the acid developed was favorable for agglutination.

Large quantities of broth were required to obtain sufficient bacteria for one experiment. Usually a 6 or 12 liter flask of broth was seeded with 50 cc. of an actively growing culture. Sedimentation was complete after 12 to 16 hours incubation at 37°C., so that the supernatant medium could be siphoned off without disturbing the bacteria. The sediment was centrifuged, washed with distilled water, and transferred with a few cubic centimeters of phosphate solution ($\frac{M}{15}$, pH 7.0) to a sterile agate mortar. The mortar contained about 2 gm. of powdered glass. It was covered with thin rubber sheeting arranged so that the bacteria could be dried and ground without contamination. The streptococci were dried in a vacuum over phosphoric anhydride. When they were nearly dry the mortar was removed from the desiccator and the bacteria were ground with the occasional addition of several drops of distilled water until only a few cell bodies were sufficiently intact to retain the gentian violet in Gram-stained films. The ground material was next pipetted into a graduate and covered with a layer of toluene. This step was necessary to kill the few bacteria remaining alive after grinding. 12 hours later the bacterial suspension was pipetted from beneath the toluene and centrifuged until clear. The supernatant fluid was made up to the volume required in each experiment with $\frac{M}{15}$ phosphate solution (pH 7.0). This volume could not be more than 50 cc. without reducing the concentration of the enzyme beyond a point where decisive results were obtainable. All the glassware and solutions used in the procedure were sterile.

The enzyme solutions were opalescent at pH 7.0 but showed a flocculent precipitate when they were titrated to pH 5.0 with lactic acid. Ordinarily they varied in reaction from pH 6.8 to 7.2. Broth cultures of the final solutions were sterile. Only Gram-negative precipitate was found in the stained films of these bacterial extracts.

Preparation of the Substrate Solution.—The substrates in the majority of the experiments were peptone, cane-sugar, or esters of fatty acids. Ordinarily the solutions were prepared so that the final concentration would be 1 per cent. For the demonstration of lipolytic action the strength of the solutions of ethyl butyrate or triacetin were such that in some of the trials this final concentration was 2 per cent. Solutions or emulsions which would withstand temperatures above 100°C. were autoclaved.

Peptone solutions (either 2 or 4 per cent in distilled water) were adjusted to pH 7.0 with sodium hydroxide and sterilized by the Arnold method. The commercial bacteriologic peptone was so completely hydrolyzed that increases in amino nitrogen could not be determined after further digestion with active trypsin. The peptone[1] employed was prepared especially for these experiments. It contained 12.5 per cent nitrogen, 16 per cent of which could be determined as amino nitrogen by Van Slyke's method. After the solutions were sterilized the amino nitrogen was increased to 20 per cent of the total.

Albumin was obtained from sterile horse serum fractioned with ammonium sulfate. The precipitate was dialyzed and concentrated in a vacuum with sterile precautions. The concentration of the concentrated solution was determined by the Kjeldahl method and then adjusted to 4 per cent. This 4 per cent solution was heated at 56°C. for 1 hour. The casein was dissolved in a neutral phosphate solution and heated for 1 hour on 3 successive days in the Arnold sterilizer. Sterile horse serum was inactivated at 56°C. for 1 hour. 4 per cent solutions of the carbohydrates in $\frac{M}{200}$ phosphate mixture were sterilized in the autoclave. Cane-sugar was autoclaved at pH 7.2, dextrose at 6.8, and starch at 7.0.

Chemical Methods.—H ion concentrations were determined colorimetrically with mixtures of sodium citrate and hydrochloric acid or of phosphates prepared according to Sörensen (1). The readings were made by the comparator method with the Clark and Lubs (2) series of indicators.

Except in instances in which the macro Kjeldahl method is specified the total nitrogen and non-protein nitrogen were determined by the micro method of Folin and Wu (3). Trichloroacetic acid was used as a precipitant in the non-protein nitrogen determinations.

The amino nitrogen estimations were made with the micro apparatus described by Van Slyke (4). Solutions of alanine and leucine were first analyzed and the results were within 0.5 per cent of the theoretical values. The peptone substrates were not precipitated before analysis.

[1] This peptone was prepared by Fairchild Bros. and Foster, New York.

The percentage of inversion of the cane-sugar was determined by the colorimetric method of Folin and Wu (5). Preliminary precipitation of the small amount of protein in the mixtures of cane-sugar and enzyme was found to be unnecessary. The results have been expressed as milligrams of dextrose per 100 cc. of final solution.

The action of the lipase was determined by the method recently employed by Avery and Cullen (6). These results have been expressed in terms of cubic centimeters of $\frac{N}{50}$ alkali required to adjust the reaction of the active to that of the inactive enzyme tubes. They are based on 100 cc. amounts of substrate.

Bacteriologic Methods.—Aside from the toluene used for the sterilization of the enzyme solutions no antiseptics were required. In the preparation of the enzyme very little toluene was apparently carried over when the bacterial emulsion was pipetted into the centrifuge tubes because the digestion flasks were occasionally contaminated. Broth cultures of these flasks were made before chemical analysis; no experiments have been included in which the most rigid tests did not show sterility throughout. The purity of the cultures from which the enzymes were obtained was proven on blood agar plates.

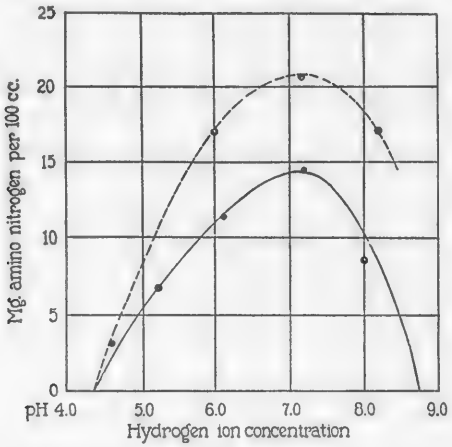
EXPERIMENTAL.

The Effect of Acid Concentration on the Action of the Peptase, Invertase, and Lipase.

Enzyme action is known to occur with the proper substrate under certain physical conditions which vary according to the nature of the enzyme. The range of acid concentration through which the reaction occurs and the optimum acid concentration are important in deciding the type of enzyme. Fuhrmann (7) noted that in a general way proteolytic bacterial enzymes responded to alkali and acid in a manner very similar to pancreatic trypsin. More recently Avery and Cullen (8) have shown that the peptase of pneumococcus has an optimum range of pH 7.0 to 7.8. This, together with the percentage of splitting obtained, led these authors to suspect that the enzyme was of an erepsin-like nature. They found likewise that the lipase (6) and invertase (9) of pneumococcus exhibited ranges similar to that of the peptonase.

Experiments 1 and 2. The Effect of Acid Concentration on the Splitting of Peptone.—Enzyme obtained from streptococci grown in 0.1 per cent dextrose broth was tested with a sterile solution of peptone as substrate. 10 cc. of the solution were pipetted into sterile 20 cc. volumetric flasks and sufficient HCl or NaOH was added to adjust the H ion concentration to the desired pH; 5 cc. of enzyme solution were added to each flask and the volume was brought up to 20 cc. with $\frac{M}{15}$ phosphate or citrate mixture of the pH desired.

Amino nitrogen determinations were done after digestion at 37°C. for 48 hours. Duplicate flasks prepared with boiled enzyme served as controls for each of the digestions. Flasks prepared without peptone served to control any increase in amino nitrogen due to the digestion of the protein in the bacterial extract. The



TEXT-FIG. 1. The effect of acid concentration on the peptone-splitting action of the enzyme solutions (Tables I and II).

object of the experiments was to determine the effect of acid concentration on the enzyme action within a sufficiently wide range to aid in establishing the identity of the enzyme. The final concentration of the peptone in the flasks was 1 per cent. Cultures made from the flasks immediately preceding analysis were sterile. The results are given in Tables I and II.

Experiments 3 and 4. The Effect of Acid Concentration on the Inversion of Cane-Sugar.—The details of these experiments are similar to those of Experiments 1 and 2 except that a solution of saccharose was used for substrate. A 4 per cent solution of cane-sugar was autoclaved in $\frac{M}{200}$ phosphate mixture at pH7.2; 5 cc. were pipetted into each 20 cc. flask so that the final concentration of the sugar was 1 per cent. The enzyme solution was divided into equal portions, one of which was boiled for 20 minutes. Duplicate flasks were prepared at the

TABLE I.

The Effect of Acid Concentration on the Splitting of Peptone.

Flask No.	Final acid concentration.		Amino nitrogen per 100 cc. of solution.		
	Active.	Inactive.	Active.	Inactive.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1, 1a	8.2	8.1	41.5	24.4	17.1
2, 2a	7.2	7.2	45.6	25.1	20.5
3, 3a	5.9	6.0	40.8	24.0	16.8
4, 4a	4.6	4.6	27.9	24.9	3.0
5, 5a*	8.0	8.0	3.3	3.6	0.0

* Flasks containing boiled and unboiled enzyme without substrate to control the autolysis of the protein in the enzyme solution.

TABLE II.

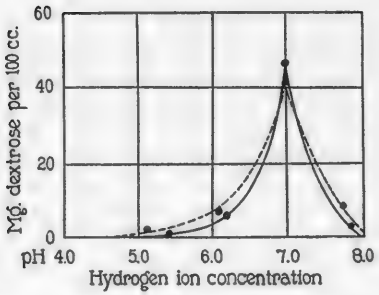
The Effect of a Greater Acid and Alkaline Range on the Splitting of Peptone.

Flask No.	Final acid concentration.		Amino nitrogen per 100 cc. of solution.		
	Active.	Inactive.	Active.	Inactive.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1, 1a	8.8	8.8	27.7	24.3	3.4
2, 2a	8.0	8.0	33.0	24.5	8.5
3, 3a	7.2	7.1	38.5	24.3	14.2
4, 4a	6.1	6.1	35.6	24.3	11.3
5, 5a	5.2	5.2	30.0	23.2	6.8
6, 6a	3.9	3.9	22.7	22.7	0.0
7, 7a	3.2	3.2	28.6	28.9	0.0
8, 8a*	7.0	7.1	3.5	3.1	0.4
9, 9a†	7.0	7.0	26.6	26.6	0.0

* These flasks contained active and inactive enzyme without peptone.

† After the supernatant fluid was removed from the centrifuge tubes in the preparation of the enzyme solution the sediment consisting of ground streptococci was washed once and divided in half. One half was boiled. Digestion tubes were prepared with this boiled and unboiled sediment and peptone.

various acid concentrations with 5 cc. of active and 5 cc. of inactive enzyme. The invert sugar was determined as dextrose by the micro method of Folin and Wu after 48 hours of incubation. Cultures from the flasks were sterile. The invert sugar has been expressed as milligrams of dextrose per 100 cc. of final solution. Two experiments have been included in Table III on account of the peculiar type of curve obtained.



TEXT-FIG. 2. The effect of acid concentration on the inversion of saccharose (Table III).

TABLE III.

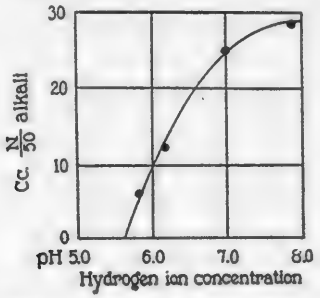
The Effect of Acid Concentration on the Inversion of Saccharose.

Flask No.	Acid concentration.			Dextrose per 100 cc. of solution.		
	Initial.	Final.		Active.	Inactive.	Increase.
	Active.	Active.	Inactive.			
	pH	*pH*	*pH*	*mg.*	*mg.*	*mg.*
1, 1a	7.8	7.8	7.8	9.5	7.5	2.0
2, 2a	7.0	7.0	7.0	53.2	7.5	45.7
3, 3a	6.2	6.2	6.2	12.1	7.4	4.7
4, 4a	5.4	5.4	5.4	8.1	7.5	0.6
1		7.7	*	15.9	*	8.6
2		7.0		49.0		41.7
3		6.1		12.8		5.5
4, 4a		5.1	5.1	10.0	7.3	2.7

* In order to concentrate the enzyme in this experiment with the hope that greater inversion might be obtained, controls with boiled enzyme were omitted from the series with the exception of the control on Flask 4. This was chosen to rule out any possibility of interpreting as a result of enzyme action, increased amounts of dextrose which might occur purely from acid hydrolysis after long incubation at pH 5.1.

Experiment 5. The Effect of Acid Concentration on the Hydrolysis of Ethyl Butyrate.—The details of this experiment are similar to those of the preceding four experiments except that ethyl butyrate was used as substrate. 5 cc. of a 4 per cent emulsion of ethyl butyrate sterilized in the Arnold sterilizer were pipetted into each of eight flasks so that the concentration was 1 per cent after the volumes were made to 20 cc. Four of the flasks were prepared with boiled

and four with unboiled enzyme solution and the volume was made up with $\frac{M}{5}$ phosphate of the desired pH. After 48 hours of incubation the active enzyme mixtures were adjusted to the pH of the similar control containing the boiled enzyme. The results have been expressed in Table IV in cubic centimeters of alkali required to adjust the pH of 100 cc. of the solution after hydrolysis to that of the control flask containing the boiled enzyme.



TEXT-FIG. 3. The effect of acid concentration on the hydrolysis of ethyl butyrate (Table IV).

TABLE IV.

The Effect of Acid Concentration on the Hydrolysis of Ethyl Butyrate.

Flask No.	Acid concentration.				Amount of N/50 NaOH required in titration.
	Inactive.		Active.		
	Initial.	Final.	Initial.	Final.	
	pH	*pH*	*pH*	*pH*	*cc.*
1, 1a	7.8	7.8	7.8	7.1	29.0
2, 2a	7.0	7.0	7.0	6.6	25.0
3, 3a	6.2	6.2	6.2	5.8	12.0
4, 4a	5.8	5.8	5.8	5.6	6.0

The effect of acid concentration on the action of these enzymes is similar to the effect observed with the enzymes of other bacteria. In this respect they are nearly identical with the endoenzymes obtained by Avery and Cullen (6, 8, 9) from pneumococci, both with regard to the range of acidity through which they are active and the optimum acid concentration.

The peptolytic enzyme (Text-fig. 1) is not active below pH 4.4 and has an optimum action at about pH 7.2. The acid limit for

the inversion of cane-sugar was about pH 5.0 and the optimum action occurred at pH 7.0. According to Text-fig. 2 the curves showing the effect of acid on inversion differ from those for peptolytic and lipolytic action. Below pH 7.0 the type of curve obtained would appear to indicate that the optimum range was narrower for the invertase than for the other enzymes; while this part of the curve is established on three determined points, the curve above pH 7.0 in Text-fig. 2 is hypothetical because there are only two determinations within this range. It is possible that with more active preparations of invertase the curve would be similar to those obtained for the lipase and the peptase. The lipase of this streptococcus has an optimum reaction more alkaline than either of the other enzymes studied (Text-fig. 3).

The Effect of Heat on the Action of the Peptase, Invertase, and Lipase.

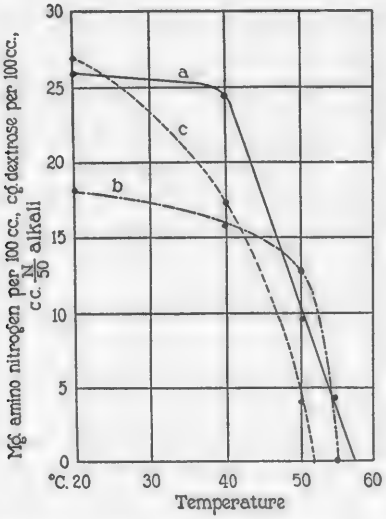
Susceptibility to heat is one of the characteristics of enzyme solutions. The temperature which destroys the enzyme varies with the source and type of enzyme and the conditions under which the heat is applied. Dry enzyme or enzyme in solution with substrate may resist temperatures which are destructive to simple aqueous solutions. The enzyme solutions in the following experiments were extracted from the streptococci without the addition of materials (with the exception of the toluene used in the procedure) foreign to bacterial growth and with a minimum amount of protein present. Neutrality was maintained during the extraction and heating. For this reason it is not possible to compare the results with those of previous workers too closely. The digestions were carried out at approximately the optimum acid concentration.

Experiment 6. The Effect of Heat on the Peptolytic Enzyme.—The enzyme solution was prepared as in the preceding experiments. It was divided among several thin walled tubes which were plunged into water of the desired temperatures for 10 minutes and afterward cooled quickly in ice water; 5 cc. of each of the heated portions were pipetted into flasks with 5 cc. of 4 per cent peptone solution. The volume of each flask was made up to 20 cc. with $\frac{M}{15}$ phosphate mixture (pH 7.0). Amino nitrogen determinations were made after incubation at 37°C. for 48 hours (Table V). The flasks were sterile after incubation. The initial and final pH of each flask was 7.0.

Experiment 7. The Effect of Heat on the Invertase.—This experiment is similar in every respect to Experiment 6 except that saccharose was used as the substrate.

Flask 1 contained enzyme which had been boiled 20 minutes. The substrate, saccharose, was autoclaved in 4 per cent solution at pH 7.2. The volume of each digestion was 20 cc. so that the final concentration was 1 per cent. The invert sugar was determined by the micro method after 48 hours incubation (Table VI). The initial pH and the final pH of each flask were 7.0. Cultures from the flasks were sterile.

Experiment 8. The Effect of Heat on the Lipase.—The procedure was similar to that in the preceding experiments. Ethyl butyrate served as substrate. A 4 per cent emulsion was sterilized in the Arnold sterilizer; 5 cc. were then pipetted into the digestion flasks with 5 cc. of enzyme solution which had been subjected



TEXT-FIG. 4. The effect of heat on the solutions of (*a*) peptase, (*b*) lipase, and (*c*) invertase (Tables V, VI, and VII).

to temperatures of 20°, 40°, 50°, and 55°C. for 10 minutes. The enzyme in Flask 1 was boiled 20 minutes. The volume of each flask was made up to 20 cc. with $\frac{M}{15}$ phosphate solution. The titrations were made with $\frac{N}{50}$ NaOH. The results are expressed in cubic centimeters of alkali required to adjust the reaction of 100 cc. of digest to that of the control flask prepared with boiled enzyme (Table VII).

The results obtained in the preceding experiments on the effect of exposing the enzyme solutions to heat have been summarized in Text-fig. 4. In order to correlate the various terms in which the results have been expressed considerable interpolation has been nec-

TABLE V.

The Effect of Heat on the Peptolytic Enzyme.

Flask No.	Temperature at which enzyme was heated for 10 min.	Amino nitrogen per 100 cc. of solution.	
		Analysis.	Increase.
	°C.	mg.	mg.
1	100*	26.9	
2	60	26.5	0.0
3	55	31.3	4.4†
4	50	36.2	9.3
5	40	51.3	24.4
6	20	52.9	26.0

* Flask 1 was prepared with enzyme which was boiled 20 minutes.

† Cultures of the streptococcus in broth were not viable after they were heated at 55°C. for 10 minutes. They were not killed at 50°C.

TABLE VI.

The Effect of Heat on the Invertase.

Flask No.	Temperature at which enzyme was heated for 10 min.	Dextrose per 100 cc. of solution.	
		Analysis.	Increase.
	°C.	mg.	mg.
1	100	7.2	
2	55	7.0	0.0
3	50	48.2	41.0
4	40	178.7	171.5
5	20	277.7	270.5

TABLE VII.

The Effect of Heat on the Lipase.

Flask No.	Temperature at which enzyme was heated for 10 min.	Acid concentration.		Amount of N/50 NaOH required in titration.
		Initial.	Final.	
	°C.	*pH*	*pH*	cc.
1	100	7.7	7.7	
2	55	7.7	7.7	0.0
3	50	7.7	7.4	13.0
4	40	7.7	7.3	16.0
5	20	7.7	7.3	18.0

essary to bring all the curves within a similar range. The temperatures required to destroy these enzymes were between 50° and 60°C. and corresponded closely with the thermal death-point of the streptococcus from which they were obtained. The results suggest that the death of the bacteria is due to the destruction of the enzymes. Such a conclusion, however, would not be in agreement with the previous literature. Repeated references are made to bacterial enzymes which are destroyed only by temperatures far in excess of that required to kill the bacteria from which they were obtained. Avery and Cullen found this to be true for the pneumococcus.

The Effect of Exposure to Acid.

The length of time during which solutions of enzyme remain active depends largely on the physical conditions under which they are preserved. In the preceding experiments temperatures above 55°C. destroyed the enzymes when they were in solution at reactions optimum for their action. There was slight deterioration even at a temperature as low as 40°. In view of this sensitiveness to heat in neutral solution, a series of experiments was devised to discover the effects of weak acid. The enzyme was exposed to lactic acid by titrating to pH 5.0, incubating at 37°C. for 6 hours, and finally adjusting the solution to the optimum acidity. Lactic acid was chosen because it is one of the principal fermentation products of dextrose in broth cultures of streptococcus (10).

Experiment 9. The Effect of Exposure to Acid.—The bacterial sediment from 12 liters of 2 per cent peptone broth was extracted in the usual manner. Dextrose was not added to this broth because it was desirable to avoid exposing the bacteria to acid during their growth. The enzyme solution was divided into three equal portions to which 10 drops of methyl red and phenolsulfonephthalein were added. One lot was boiled for 20 minutes; the second portion was adjusted to pH 5.0 with lactic acid; the third served for a control. All three were incubated at 37°C. for 6 hours. The acid tube was titrated to pH 7.0 (the acid concentration which had been maintained in the other tubes during incubation) with NaOH at the end of this period. All three tubes were then made up to equal volumes with phosphate mixture (pH 7.0, $\frac{M}{15}$). The action of these lots of enzyme was observed with substrates of peptone, cane-sugar, and triacetin. The triacetin (8 per cent emulsion) was autoclaved at pH 7.0; the other substrates were prepared as in the preceding experiments. 5 cc. of substrate and enzyme solution were pipetted into

digestion flasks and made up to 20 cc. volume with phosphate solution. The analyses were made after incubation at 37°C. Cultures from these flasks showed no growth. The results are shown in Table VIII.

TABLE VIII.

The Effect of Exposure to Acid.

(*a*) *Peptolytic Enzyme.*

Flask No.	Treatment of enzyme.	Acid concentration.		Results per 100 cc. of solution.	
		Initial.	Final.	Amino nitrogen.	Increase.
		pH	*pH*	*mg.*	*mg.*
1	Boiled 20 min.		7.1	33.7	
2	Incubated at 37°C. for 6 hrs. at pH 5.0 and adjusted to pH 7.0.		7.1	49.8	16.1
3	Normal active enzyme.		7.1	63.9	30.2

(*b*) *Invertase.*

Flask No.	Treatment of enzyme.	Acid concentration.		Results per 100 cc of solution.	
		Initial.	Final.	Dextrose.	Increase.
		pH	*pH*	*mg.*	*mg.*
4	Boiled 20 min.	7.0	7.0	6.3	
5	Incubated for 6 hrs. at pH 5.0 and adjusted to pH 7.0.	7.1	7.1	6.4	0.1
6	Normal active enzyme.	7.1	7.1	44.0	37.7

(*c*) *Lipase.*

Flask No.	Treatment of enzyme.	Acid concentration.		Amount of N/50 NaOH required in titration.
		Initial.	Final.	
		pH	*pH*	*cc.*
7	Boiled 20 min.	7.0	6.8	
8	Incubated 6 hrs. at 37°C. at pH 5.0 and adjusted to pH 7.0.	7.0	6.8	0*
9	Normal active enzyme.	7.0	6.2	94.0

* On account of the partial hydrolysis of the inactive triacetin control the flasks were adjusted to pH 6.8. Controls without enzyme demonstrated that after triacetin had been autoclaved the reaction was more acid after 48 hours by pH 0.1 to 0.3.

Experiment 10. The Effect of Exposing Weak Solutions of Peptolytic Enzyme to Acid.—The preceding experiment was repeated with a weak solution of enzyme. The procedure was otherwise similar. The acid-treated enzyme was tested only with a substrate of peptone. The solution with which Experiment 9 was carried out yielded a heavy precipitate when it was acidified with acetic acid. The enzyme in this experiment did not show a precipitate but merely opalescence after the addition of weak acetic acid. The results of the experiment are shown in Table IX.

TABLE IX.

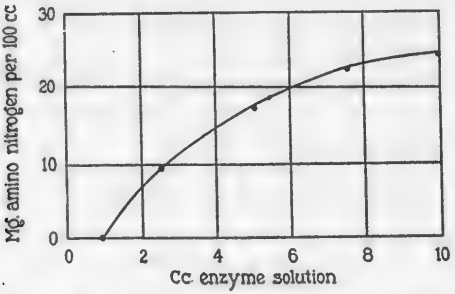
The Effect of Acid on Weak Solutions.

Flask No.	Treatment of enzyme.	Final acid concentration.	Amino nitrogen per 100 cc. of solution.	
			Analysis.	Increase.
		pH	*mg.*	*mg.*
1	Boiled 20 min.	6.9	33.5	
2	Incubated 6 hrs. at pH 5.0 and adjusted to pH 7.0.	7.0	37.4	3.9
3	Normal active enzyme.	7.0	48.0	14.5

These experiments demonstrate that two of the enzymes are exceedingly susceptible to acid and that the third, the peptase, is partially destroyed at pH 5.0. It is difficult to account for the selective action of acid aside from the protective action which the protein in the solution might have for the peptase. Analyses show that the protein was far in excess of the percentage of reducing substances found in these solutions and for that reason it is logical to suppose that it protected the peptolytic enzyme. The invertase was probably more easily destroyed by acid in the absence of substrate or digestion products. The percentage of fat in the solutions was not estimated. In the second of these experiments the enzyme solution was less concentrated and contained but little protein; the deterioration of the enzyme was considerably greater. There is, of course, no objection to interpreting these results as due to actual differences in the character of the enzymes, since it was impossible to obtain purer solutions for further experiments.

Experiment 11. The Effect of Enzyme Concentration on Peptolytic Digestion.—The enzyme and substrate were prepared as in the preceding experiments. Flasks were prepared with 5 cc. of 4 per cent peptone solution and increasing

amounts of enzyme. The volumes were made to 20 cc. with $\frac{M}{15}$ phosphate mixture. Analyses were made after 48 hours of incubation at 37°C. (Table X and Text-fig. 5).



TEXT-FIG. 5. The effect of concentration of enzyme on the splitting of peptone (Table X).

TABLE X.

The Effect of Concentration of Enzyme on the Splitting of Peptone.

Flask No.	Amount of enzyme.	Final acid concentration.		Amino nitrogen per 100 cc. of solution.		
		Active.	Inactive.	Active.	Inactive.	Increase.
	cc.	*pH*	*pH*	*mg.*	*mg.*	*mg.*
1	10.0	7.2	7.2	52.1	28.3	23.8
2	7.5	7.1	7.2	50.9	28.7	22.2
3	5.0	7.2	7.2	45.5	28.3	17.2
4	2.5	7.1	7.1	38.0	28.4	9.6
5	1.0	7.2	7.2	28.4	28.7	0.0

Table X shows that the weaker concentrations of enzyme are more effective in digesting peptone than are more concentrated solutions. Bayliss (11) has shown that the time element is important in concentration studies but concludes that weight for weight, regardless of the type of velocity curve which digestion with enzymes may show, higher concentrations are more effective than the lower ones. In this respect, it is apparent that this peptase follows the general law of enzymes.

Experiment 12. The Proteolytic Action of the Enzyme Solutions.—The solution of enzyme was prepared in the usual manner from 6 liters of 0.1 per cent dextrose broth culture of streptococci. The serum albumin was obtained by fractioning

sterile horse serum with ammonium sulfate, then dialyzing, and concentrating the dialyzed solution in vacuum; this procedure was carried out without bacterial contamination. The solution was heated to 56°C. for 1 hour before it was used. The casein solution was sterilized in the Arnold sterilizer. The horse serum was inactivated at 56°C. The solutions were prepared in 4 per cent concentrations; the peptone and casein were weighed but the albumin and horse serum were diluted until they contained 4 per cent protein reckoned on the basis of the total Kjeldahl nitrogen. This, of course, introduced a slight error in the protein concentration of the diluted horse serum. The solutions were adjusted to pH 7.0 with sterile acid or alkali.

5 cc. of enzyme solution, 5 cc. of substrate, and 10 cc. of phosphate solution were pipetted into sterile flasks. These mixtures were incubated at 37°C. for 3 days and analyzed by the micro Kjeldahl method after precipitation with trichloroacetic acid (Table XI). Duplicate and triplicate precipitations with 10 per cent trichloroacetic acid with solutions of various proteins demonstrated that the precipitation was equal and constant.

TABLE XI.

The Proteolytic Action of the Enzyme Solutions.

Flask No.	Substrate.	Final acid concentration.		Non-protein nitrogen per 100 cc. of solution.		
		Active.	Inactive.	Active.	Inactive.	Increase.
		pH	*pH*	*mg.*	*mg.*	*mg.*
1	Casein.	6.9	6.9	25.6	12.6	13.0
2	Albumin.	6.9	6.9	15.5	15.1	0.4
3	Serum.	7.0	7.0	21.1	19.3	1.8
4	Peptone.*	6.9	6.9	63.8	28.0	35.8

* The peptone digestion was included as a control on the activity of the enzyme solution. These results are expressed in milligrams of amino nitrogen per 100 cc. of solution.

The results obtained in the preceding experiment indicate that the enzymes have but slight ability to bring about the digestion of higher proteins. The increase in non-protein nitrogen in the albumin-enzyme mixture is so small that it may have resulted either from autolysis of proteins in the enzyme solution or from an experimental error in the determinations. A series of determinations with solutions of enzyme showed increases of 0.1 to 0.2 mg. of non-protein nitrogen per 100 cc. of solution after active bacterial extracts were allowed to autolyze for several days. This increase, however, is so slight that

it need hardly be considered. The digestion of casein is definite since the increase was 13 mg. in 100 cc. of digest; this indicates that over 8 per cent of the protein was attacked. The failure of the enzyme to digest serum albumin might be due either to an antienzymatic action of this protein or to an inability of the enzyme to attack it. In view of the increase in the non-protein nitrogen of the serum after digestion, an increase which may be explained by the splitting of protease normally found in serum in small amounts, it seems probable that there is little inhibition of the enzyme by the serum albumin. Such action on casein and failure to digest albumin are characteristic of the erepsin-like enzymes previously found in many plants (12). From the tables given by Avery and Cullen in their study of the proteolytic action of the pneumococcus endoenzymes it is apparent that the "peptase" which they obtained acted similarly on casein and egg albumin, since they were unable to obtain mixtures beyond that due to the autolysis of the proteins occurring in the enzyme solutions alone.

The Effect of Chloroform and Gentian Violet on the Peptolytic Enzyme.

Early in these experiments it was found necessary to employ an antiseptic in the preparation of the bacterial extracts to kill the few bacteria which escaped grinding. Since toluene was known to have a definite bactericidal action and had been used generally to preserve solutions of organic material or to prevent bacterial growth during digestion with enzymes, it was used throughout these experiments in preparing the solutions. It was felt that other antiseptics might be used which would serve a similar purpose. A series of experiments was than carried out to study the effects of chloroform and gentian violet.

Experiment 13. Comparison of the Effects of Toluene and Chloroform on the Peptolytic Enzyme.—An emulsion of the bacteria was prepared in the usual manner. After the mass was well ground half was allowed to stand with toluene and half with chloroform for 12 hours. Each half was centrifuged after that period and the supernatant solutions were pipetted from the tubes without disturbing the layers of antiseptic. The solutions were sterile. Digestion flasks were prepared with substrates of peptone. Controls containing enzyme which had been boiled 20 minutes were included in the series. After 48 hours at 37°C. the amino

nitrogen was determined for each flask (Table XII). Cultures made in broth showed no growth.

TABLE XII.

The Effect of Chloroform on the Peptolytic Enzyme.

Antiseptic.	Amino nitrogen per 100 cc. of solution.		
	Active.	Inactive.	Increase.
	mg.	*mg.*	*mg.*
Toluene	53.9	26.1	27.8
Chloroform	34.6	28.9	5.7

Experiment 14. The Inhibition of the Peptolytic Action of Enzyme Solutions by Gentian Violet.—Solutions of enzyme and peptone were prepared with toluene in the usual manner. Dilutions of gentian violet (Grübler) were made in $\frac{M}{15}$ phosphate solution (pH 7.0). 10 cc. of each of these dilutions were pipetted into sterile flasks with 5 cc. of enzyme and 5 cc. of 4 per cent peptone solution. Amino nitrogen determinations were made after 48 hours at 37°C. (Table XIII). Controls without gentian violet were prepared with boiled and normal active enzyme.

TABLE XIII.

The Effect of Gentian Violet on the Peptolytic Enzyme.

Flask No.	Dilution of gentian violet.	Precipitate.*	Amino nitrogen per 100 cc. of solution.	
			Analysis.	Increase.
			mg.	*mg.*
1	1:1,000	++++	29.8	3.0
2	1:5,000	+++	37.9	11.1
3	1:10,000	++	43.8	17.0
4	1:20,000	+	43.6	16.8
5	1:50,000	+	56.8	30.0
6	1:100,000	+	57.0	30.2
7	Active normal enzyme.		56.8	30.0
8	Inactive enzyme.		26.8	

* Precipitate was formed in these flasks during incubation; the amount of precipitate has been indicated by plus signs.

Previous workers have observed that chloroform has an inhibiting action on digestion with enzyme; this has been noted frequently in the literature with respect to pancreatic trypsin. According to Fuhrmann, van Laer observed that solutions of diastase were inactive

if they were preserved with chloroform for more than 15 days. Fuhrmann (7) furthermore noted that chloroform was detrimental to bacterial enzyme action, while toluene was one of several antiseptics which might be used for the sterilization of cultures to demonstrate enzyme action. The results of Experiment 14 serve merely as confirmatory evidence of these facts. Although sterilization was accomplished with both of the antiseptics the use of chloroform resulted in considerable deterioration of the solutions.

The effects of the dilutions of gentian violet on the splitting of peptone were more surprising because Churchman (13) found that this dye had no effect on the digestion of proteins with trypsin. A very definite decrease in the peptolytic activity of these enzyme solutions was found in dilutions as high as 1:20,000. In 1:1,000 dilution the enzyme was practically inactive. Slight precipitation occurred throughout the series of flasks to which gentian violet had been added, but was most marked in dilutions more concentrated than 1:20,000. Since precipitates were not obtained with solutions of pure albumin or with the enzyme solution in similar dilutions of the dye, a series of tubes was prepared with 1 per cent of the peptone used in the experiment.

Precipitate occurred in approximately the same amounts as had been found in the enzyme-peptone mixtures. From nitrogen determinations on the supernatant fluid obtained by centrifuging these peptone mixtures it was found that nearly one-tenth of the peptone was carried out of solution in a 1:1,000 dilution. The degree of precipitation in the various dilutions corresponded roughly to the inhibition of the enzyme in similar concentrations of the dye. In dilutions of 1:50,000 and 1:100,000, which will ordinarily inhibit Gram-positive bacteria, digestion was not hindered. These observations are not sufficiently comprehensive so that one may offer an entirely satisfactory explanation for the bacteriostatic action of gentian violet. It is known that violet-positive bacteria (14) will absorb sufficient dye during growth on agar containing almost imperceptible quantities so that the growing colonies acquire a deep purple color, and that greater percentages will entirely inhibit growth. These facts suggest that its bacteriostatic effect may be due to the absorption of sufficient quantities of dye to inhibit enzyme action.

Evidently, since the trypsin digestion of proteins is not hindered by gentian violet, the inhibition is not due to a direct action on the enzyme. Furthermore, only one-tenth of the peptone was carried out of solution by the dye so it is not probable that the digestion was inhibited to any great extent by a change in the concentration of the substrate. The most plausible explanation is an absorption of the enzyme by the precipitate which might occur in Gram-positive bacteria when exposed to gentian violet as well as in the peptone-enzyme mixtures.

DISCUSSION.

The method used to extract the enzymes from the streptococci is a combination of various procedures which have been employed by previous investigators. Various innovations were introduced, such as the use of the phosphate mixtures during the grinding and sterilization of the cultures to protect the enzymes from deterioration. Further work showed that the three enzymes studied were exceedingly susceptible to heat, acid, and antiseptics, so that all the attempts made to protect them during extraction were justified. A method was desired which might be applied to other bacteria and would yield solutions of enzyme relatively free from cell debris. Although considerable protein was carried into solution the consistent results which were obtained throughout the experiments lead us to suspect that the physical conditions under which the various digestions were studied were not greatly modified by the material dissolved from the cell bodies. No attempt was made, however, to purify the solutions further.

The methods of study are similar to those previously employed by Avery and Cullen (6, 8, 9) in their work with pneumococcus, except that micro methods of analysis have been employed when possible in preference to methods requiring larger quantities of material for analysis. The enzymes resemble those which Avery and Cullen obtained from the pneumococcus, since they act through similar ranges of H ion concentration. The resemblance between the enzymes leads us to believe that similar enzymes exist in other bacteria.

The experiments on the effects of acid concentration showed that the enzymes studied were most active between pH 7.0 and 8.0. This

pH corresponds closely with the degree of alkalinity necessary for the optimum growth of streptococci. The lipase was most active at a pH more alkaline than that required for the other enzymes; this appears to be true for lipase obtained from other than bacterial sources. The acidity at which digestion might proceed varied. The lipase was found inactive at a point less acid than the final pH of cultures in dextrose broth, but the peptase, according to the curve constructed in Text-fig. 1 was active at an acid concentration slightly greater than pH 4.5. The limits of digestion in acid probably depend both on a union of substrate and enzyme and on the destruction of the enzyme itself; some of the later experiments on the effect of acid serve to elucidate this point. In Experiment 9 an acid concentration of pH 5.0 was found to be sufficient to destroy the invertase and lipase if solutions were exposed to the acid for 6 hours at a temperature of 37°C., while the peptolytic enzyme was still active after similar treatment. This suggests that the digestion obtained with the peptase in acid concentrations which completely inhibited the lipase and the invertase may be due to the resistance of that enzyme to acidities as low as pH 5.0.

Further work on physical conditions destructive to the enzymes was continued in Experiments 6, 7, and 8. A temperature of 60°C. continued for 10 minutes was found to be sufficient to destroy all three enzymes. According to the curves in Text-fig. 4, there was some deterioration even at 40°C., and the temperatures required for complete destruction were between 51° and 57°C. The streptococci from which the extracts were obtained were not viable after cultures were heated for 10 minutes at 55°C. The close agreement between these temperatures suggests that the destruction of the enzymes may be one of the factors causing the death of the organism by heat. The great number of previous observations on this point is hardly compatible with such a conclusion, since repeated observations by other investigators indicate that bacteria are ordinarily killed by temperatures which will not destroy their enzymes. Fuhrmann, however, observes that solutions of enzyme obtained from bacteria grown on media relatively free from protein are destroyed at lower temperatures than enzymes obtained from cultures on ordinary media. Other observations with enzymes from non-bacterial sources indicate that

products of digestion have a distinctly protective action. Throughout these experiments the peptase was the most stable of the enzymes. Analyses indicate that the solutions of enzyme contained considerable protein material; there was relatively little reduction of alkaline copper solutions, so we assume that there was a much smaller percentage of dextrose. It is probable that the amounts of protein and sugars in the solution account for the difference in stability of the enzymes. Such an assumption would explain the higher temperatures required to destroy the enzymes of pneumococci obtained by the complete solution of the bacterial bodies with the resulting autolysis and accumulation of digestion products.

Efforts were made to ferment dextrose by the addition of freshly ground bacteria and of yeast coenzyme to the digestion mixtures. No changes in acidity were observed after these procedures. Starch was not attacked by the enzyme solutions.

CONCLUSIONS.

1. A method has been outlined by which the enzymes of hemolytic streptococcus may be extracted with comparative ease.

2. The peptolytic enzyme is active between pH 4.4 and 8.7 with an optimum action at pH 7.2. It is destroyed in neutral phosphate solution at a temperature of 57°C. continued for 10 minutes and at pH 5.0 deteriorates slowly at 37°C. Concentration experiments with solutions of the enzyme have shown that it resembles other enzymes. It is exceedingly susceptible to chloroform and its action is inhibited by dilutions of gentian violet. Casein is attacked but serum albumin is not digested after 3 days at 37°C.

3. The invertase is active between approximately pH 5.0 and 8.0 with an optimum of pH 7.0. It is destroyed by a temperature of 52°C. continued 10 minutes at an acid concentration of pH 7.0, or after 6 hours at 37°C. at pH 5.0. At this acidity it is more susceptible to heat than the peptase.

4. The lipase is active above pH 5.6. The greatest activity was observed at pH 7.9. It is completely destroyed after heating to temperatures over 55°C. for 10 minutes and resembles the invertase in its susceptibility to acid.

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STUDIES ON EXPERIMENTAL PLETHORA IN DOGS AND RABBITS.*†

By EDWARD B. KRUMBHAAR, M.D., Ph.D., and ALFRED CHANUTIN, Ph.B.

(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania and the Laboratories of the Philadelphia General Hospital, Philadelphia.)

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The object of the present communication is to present the functional changes produced by repeated transfusions in the blood-making and blood-destroying apparatus and in metabolism, and also the structural changes in the viscera of dogs and rabbits. The source of an anemia that developed during the plethoric stage has also been considered. In an attempt to throw further light on the relation of the spleen to blood formation and blood destruction, we first studied the effect of splenectomy in artificial plethora, and tried to find evidence of increased enzyme action in the spleen removed at a time when blood was being destroyed in greatly increased quantities. Not only were these efforts barren of results, but it was also found that our knowledge of the changes caused by the artificial induction of plethora was in itself meager.

I.

Blood Formation and Destruction.

A few writers have studied the effect of plethora on rabbits, animals notoriously inconstant in their hemopoietic reactions. Hess (1) induced plethora in rabbits by repeated transfusions in order to study the effect on the work of the heart, and Itami (2) studied the tissue changes in these same animals. These writers also described an extremely plethoric rabbit, which, in spite of continued transfusions and of the absence of isolysins, developed a marked anemia. Robertson (3) has described more thoroughly this type of plethoric anemia and has shown

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† Read before the American Society for Experimental Pathology, New Haven, December 29, 1921.

the relationship of plethora and anemia to bone marrow activity, as indicated by the percentage of reticulated red cells. This writer found that agglutinins developed coincidentally with the anemia, but did not give quantitative details of the results. Kambe and Komiya (4) also encountered this anemia in rabbits that had been made plethoric and also demonstrated the presence of agglutinins qualitatively.

Ottenberg and Thalhimer (5) found that isohemolysins are seldom present in the blood of normal cats, but that they often appear in the blood of recipient cats after transfusions. With the development of hemolysins, the animals developed a marked anemia, from which they quickly recovered after cessation of transfusions. Ottenberg, Kaliski, and Friedman (6) demonstrated the presence of isoagglutinins and isohemolysins in dogs. These authors also showed that direct transfusion of blood that is agglutinated and laked by the recipient's blood is followed by the destruction of the transfused blood with an intense intoxication. Boycott and Douglas (7, 8) observed that dogs do not destroy cells during the days immediately following a first transfusion, but that once the animal has become accustomed to destroying blood, it can dispose of the transfused red blood cells two to eight times as fast as after the first transfusion. They therefore think that some mechanism must be developed by the animal for the rapid destruction of foreign red cells. Since Boycott and Douglas could find no evidence of isohemolysins after the transfusions, they concluded that transfused red cells were not destroyed by direct hemolysis.

Methods.

Plethora was produced in dogs by daily transfusions of 25 to 200 cc. of whole blood taken from six compatible donors in rotation. Dogs weighing from 6 to 10 kilos were used as recipients and larger animals as donors. With careful technique the jugular veins were used during the entire experiment for aspiration of blood from the donor and injection into the recipient. The donor's blood was drawn into an Erlenmeyer flask containing 2 cc. of 10 per cent sodium citrate for every 100 cc. of blood, to prevent clotting. The blood was allowed to flow into the recipient from a sterile burette by gravity. The hemoglobin and the total blood volume figures were used for estimating the degree of plethora.

Hemoglobin was determined by the Newcomer (9) method, and the vital red method of Keith, Rowntree, and Geraghty (10) was used in the estimation of the blood volume. The method of Wilbur and Addis (11) was employed for the determination of urobilin as an index of blood destruction.

Bone marrow activity was studied by estimating the percentage of reticulated or "skeined" erythrocytes. It is suggested that for the sake of brevity, the word "reticulocyte" be substituted for "reticulated erythrocyte," and the term "reticulosis" be designated to replace some such expression as "an increase in the number of reticulated cells," as is now the custom. The percentage of reticulocytes was estimated after vital staining with Grübler's brilliant cresyl blue.[1] The recipients were fed a standard diet of beef heart, cracker meal, sugar, lard, bone ash, and salt, and the donors were given table scraps. The test animals gained in weight during the experiment, while the donors became only slightly anemic with only a small rise in the percentage of reticulocytes. Removal of the spleen was performed under ether anesthesia without noteworthy loss of blood.

RESULTS.

Production of Plethora.

Seven dogs were rendered plethoric by the method described. The degree of plethora obtained did not depend on the amount of blood injected; for instance, one dog given twenty transfusions, averaging 48 cc., attained as great a rise in hemoglobin as another injected twenty-three times, each transfusion averaging 157 cc. The largest number of transfusions in one animal was 61 in 76 days; the shortest period was 15 days with eleven transfusions in a fatal case. The maximum hemoglobin obtained in these plethoric animals varied from 146 to 226 per cent (Text-figs. 1 to 4).

A peculiar result was noted in three out of four dogs (Nos. 1, 2, and 3) when transfusions were discontinued during the height of plethora; namely, a temporary rise in hemoglobin of 11, 17, and 21 per cent, occurring on the 3 following days. This was also noted by Boycott and Douglas in one of their rabbits. The drop in hemoglobin after the sudden rise was almost as striking. The fourth dog (No. 4) maintained a constant hemoglobin level for 10 days before the transfusions were stopped, and upon ceasing injections, there was a sudden drop. It is suggested that possibly some organ, such as the liver, acts as a blood storehouse, and upon cessation of transfusion, a further rise in

[1] American preparations of this stain have not been of any use in our hands.

blood count is caused by excess blood being thrown into the blood stream.

The results of our investigations have shown that the excess blood is destroyed as rapidly by the splenectomized as by the normal dogs, after ceasing the transfusions. Three dogs were made plethoric, their spleens removed, and transfusions stopped; the fourth dog used for a control was made plethoric but was not splenectomized. In all four cases, after discontinuing transfusions, the blood was destroyed with approximately equal rapidity, as indicated by hemoglobin and blood volume studies. After 4 to 6 weeks the hemoglobin had returned to normal and then a moderate anemia (hemoglobin varying from 15 to 27 per cent below normal) persisted for a few weeks, followed by a permanent return to normal. This anemia might be considered as an expression either of the time required by the bone marrow to recover from its enforced inactivity, or more probably of the organism's attempt to rid itself of its newly acquired powers of increased blood destruction.

The first few transfusions raise the hemoglobin percentage only very slightly. The reactions of animals during this time differ widely. Dog 3, for example, was transfused with 620 cc. of blood in 4 days, causing the hemoglobin to rise from the normal of 86 per cent to only 98 per cent. Another dog's (No. 2) hemoglobin increased only 9 per cent by the 3rd day after 95 cc. of blood had been transfused. The organism can apparently handle this excess blood during the first few days of transfusions without showing any signs of marked increased blood destruction, as will be shown below in the urobilin and metabolism studies.

One apparently successful attempt was made to break down an animal's blood-destroying mechanism (No. 5) by transfusing relatively massive quantities of blood (averaging 133 cc. per day). It was possible, by this means, to obtain a rise in hemoglobin from 98 per cent to 200 per cent in 11 days. With the continuance of these large daily transfusions, however, this very high blood concentration was after 8 days reduced by the organism to 150 per cent. Continued transfusions then apparently killed the animal after a second rise in hemoglobin to 226 per cent. The red cell count, at this time, was 15,500,000, as compared with the normal of 7,160,000. The cause of death of this animal will be considered later.

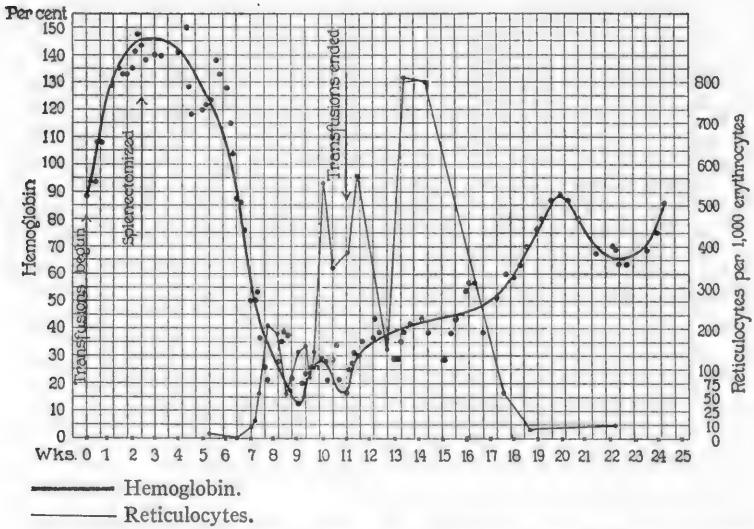
Anemia Following Plethora.

This anemia, developing in spite of and probably on account of continued transfusions, is characterized by a marked fall in hemoglobin and red blood cells with at first little reticulocyte evidence of regeneration. In one splenectomized dog (No. 6, Text-fig. 1), the anemia developed rapidly until, at the end of 15 days, the hemoglobin had fallen from 138 per cent to 21 per cent; there was then a temporary rise for several days, followed by a fall to 13 per cent, when it reached the point of greatest severity. Although transfusions were continued daily at this time, the hemoglobin at the end of 13 days was 17 per cent. The injections were stopped and the hemoglobin returned to normal slowly. In another animal (No. 7, Text-fig. 3), in which transfusions were continued after splenectomy, the hemoglobin fell from 185 per cent to 67 per cent during a period of 18 days; with continued transfusions, however, the hemoglobin rose again to a high figure.

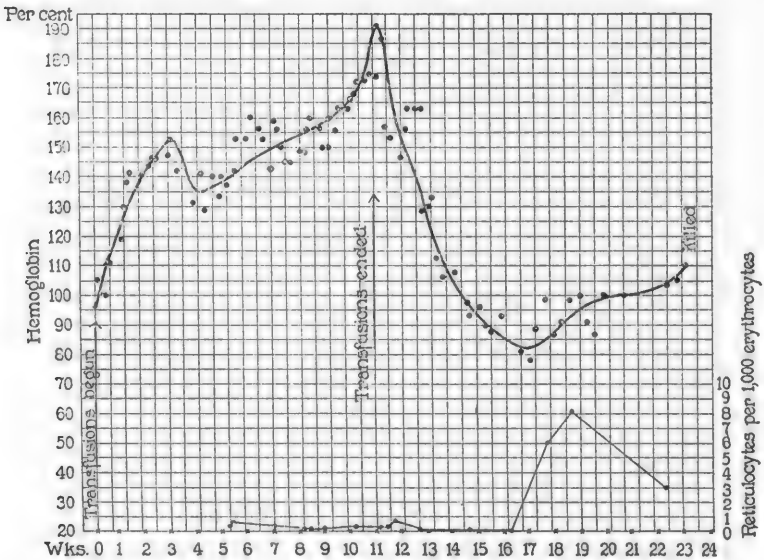
The experiment with Dog 6 was controlled by transfusing equal amounts of blood from the same donors into a normal dog (No. 4). This animal developed an artificial plethora (which persisted until transfusions were discontinued) without anemia (Text-fig. 2). The significance of this disparity will be discussed later.

Bone Marrow Activity in Plethora and Anemia.

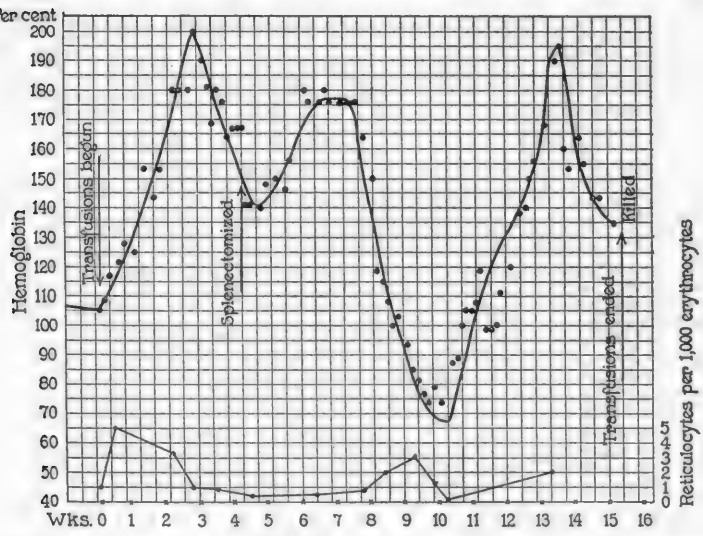
Our experiments with dogs have confirmed Robertson's (3) findings in rabbits that reticulocytes practically vanished during the stage of artificial plethora, but were greatly increased during the "plethoric anemia" stage (Text-figs. 1 to 4). The value of reticulocytes as an index of bone marrow activity and blood formation is especially evident in Text-fig. 1, which shows the sudden anemia following plethora in Dog 6. The sudden fall in hemoglobin is seen to be accompanied by a slight increase in reticulocytes, which had been practically absent in the plethoric stage. This reticulosis became marked after the anemia had been in progress 2 weeks, and after transfusions had been stopped, the reticulocytes increased to the extremely high level of 81 per cent. The animal's hemoglobin and reticulocytes gradually returned to normal after transfusions


Per cent
Hemoglobin
Reticulocytes per 1,000 erythrocytes
Transfusions begun
Splenectomized
Transfusions ended
Wks. 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Hemoglobin.
Reticulocytes.

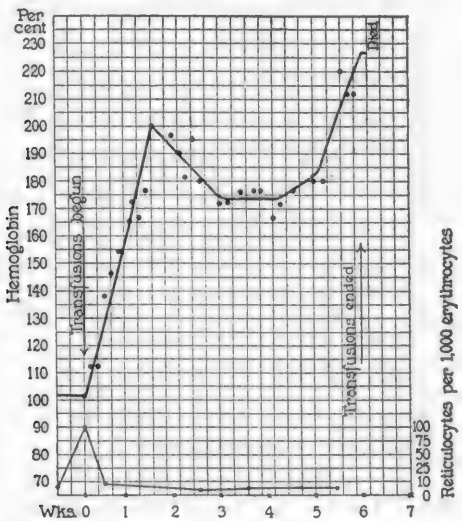
TEXT-FIG. 1. Dog 6. Reticulocyte and hemoglobin curves in experimental plethora and "plethoric anemia." The amount of blood transfused was 5,965 cc.


Per cent
Hemoglobin
Reticulocytes per 1,000 erythrocytes
Transfusions begun
Transfusions ended
Killed
Wks. 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

TEXT-FIG. 2. Dog 4. Reticulocyte and hemoglobin curves in experimental plethora. The amount of blood transfused was 6,150 cc.



TEXT-FIG. 3. Dog 7. Reticulocyte and hemoglobin curves in experimental plethora and "plethoric anemia." The amount of blood transfused was 10,450 cc.



TEXT-FIG. 4. Dog 5. Reticulocyte and hemoglobin curves in fatal experimental plethora. The amount of blood transfused was 5,000 cc.

had been discontinued. In Dog 7, in which a slight anemia developed after splenectomy, the slight increase in reticulocytes should be noted in passing.

Blood Volume Studies.

The vital red method of Keith, Rowntree, and Geraghty gives, according to Smith, Arnold, and Whipple (12), an accurate index of plasma volume, but is less reliable for red cell volume determinations. One can, however, obtain comparable results when working on the same animal for a long period of time. Keith, Rowntree, and Geraghty (10) observed in many anemic patients an increase of plasma volume and found that in polycythemia the plasma volume may be either low or high—in the latter case true plethora may be said to exist. Bock (13), on the other hand, studying the blood volume in pernicious anemia, has shown that as long as the hemoglobin remains above 30 per cent, there is a remarkable constancy of plasma volume. In patients with lower hemoglobin, there is a corresponding reduction of the plasma volume. The variations in blood volume are, for the most part, due to changes in red cell content. Denny (14) also finds that the plasma volume is essentially normal in pernicious anemia, and a decrease in total volume is due to loss of cell mass. The constancy of the plasma volume is also shown by Peters and Rubnitz's (15) report that the plasma nitrogen has no tendency to decrease in anemia.

In four dogs studied, the total blood volume was, as might be expected, greatly increased above normal in the plethoric stage and decreased below normal in the anemic stage (Tables I to IV). With the exception of one animal (No. 5), which died from the induced plethora, the plasma volume showed surprisingly few changes, either in anemia or plethora. The changes in red cell volume were chiefly responsible for the variations in total blood volume, so that the plethora might properly be termed "acythemic plethora."

It is interesting to note, in agreement with Bock's and Denny's findings, that in the extreme anemia obtained in Dog 6, the total red cell volume was estimated, once at 55 cc. and 5 days later at 37 cc., while the plasma volume remained practically normal. The total blood volume dropped from a normal of 980 cc. to a minimum of 512 cc. The anemia in Dog 7 was manifested by a change from the normal blood volume of 1,000 cc. to 794 cc. The plasma volume in this dog during the anemic period was identical with the normal, with a diminished red cell volume.

With extreme plethora (No. 5) there was a marked diminution in plasma volume, together with an increased red cell volume. This diminution of plasma volume is present to a slight extent in all cases of extreme plethora during the early stages of transfusions. There is then manifested a tendency for the plasma volume to return to the original level.

Urobilin Excretion.

The amount of urobilinogen and urobilin in the urine and stools is now generally recognized to be a fairly accurate index of the degree of blood destruction in the body (Wilbur and Addis (11) and Robertson (16)). As the experimental animals were on a constant diet throughout the experiments, Whipple's (17) demonstration that bile pigment elimination can be greatly changed by modification of diet does not apply to these experiments.

Urobilin was found to be present in the urine in small amounts only during periods of marked blood destruction. Urobilin determinations were made on the feces of three dogs (Nos. 4, 6, and 7), the first two of these having received equal amounts of blood from the same donors. Dog 4 reached a maximum urobilin excretion of 8,000 units at the same time that the plethora reached its maximum. At this point transfusions were stopped, and both blood count and urobilin excretion returned gradually to normal. Dog 6 showed a maximum output of 14,400 units at the height of the "plethoric anemia." This high urobilin output continued for about 4 weeks. When transfusions ended, and the dog's hemoglobin began to rise, accompanied by a simultaneous reticulosis, the urobilin excretion was greatly diminished. Dog 7 also showed a greatly increased urobilin elimination but press of work did not permit many determinations at this time. (Tables I to III.)

Blood Counts.

Hemoglobin and erythrocyte determinations have already been considered. Leucocytes, estimated during the course of the experiments, were considerably increased, chiefly due to an increase of polymorphonuclear leucocytes; but changes were so inconstant and unexplainable that they will not be further considered. Fragility

tests made on several animals during the course of the experiments showed an increase in both the maximum and minimum resistance, both during the plethoric and anemic stages, but these were not

TABLE I.

Blood Changes in Experimental Plethora and "Plethoric Anemia."
Dog 6.

Length of time after first transfusion.	Hemoglobin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Urobilin units.	Remarks.
days	*per cent*	*millions*	*cc.*	*cc.*	*cc.*		
(Normal.)	87		545	435	980	400	Mar. 9, 1920. Transfusions begun.
5	116		580	640	1,220	600	
8	128		645	925	1,570		
14	135		515	1,095	1,610	1,920	Splenectomized.
21	140		445	865	1,310		
23	140		427	905	1,332		
35	120		512	798	1,310		
37	123		475	775	1,250		
40	133		520	780	1,300		
45	87	4.6	583	373	956	2,400	
49	50	3.5	590	175	765		
51	53	2.5	560	115	675	11,200	
53	26	1.8	607	54	661		
57	27	1.2	640	70	710		
63	13	0.65	580	55	635	14,400	
68	26	1.39	475	37	512	13,000	Injections ended.
77	17	1.71				11,300	
78	25	1.55					
95	39	1.97	437	110	547		
102	39	2.6				3,120	
112	57	2.88	535	230	765		
122	52	3.1	550	194	744		
127	59	3.8	568	210	778	640	
158	64	3.68	568	190	758		
166	69		578	272	850		
171	87		510	300	810		
215	82	5.6					

5,965 cc. of blood transfused in 60 injections; average 99 cc. per transfusion.

sufficiently striking or constant to be given in detail. Except for the slight increase in resistance of the slightly anemic donor's cells, it is difficult to see why such a change in the fragility of the blood of

the experimental animals should have taken place during plethora. A possible explanation would be that with the increased need for

TABLE II.

Blood Changes in Experimental Plethora.
Dog 4.

Length of time after first transfusion.	Hemoglobin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Urobilin units.	Remarks.
days	*per cent*	*millions*	*cc.*	*cc.*	*cc.*		
(Normal.)	105		580	448	1,028	300	Mar. 9, 1920. Transfusions begun.
7	111		540	880	1,420	1,200	
14	141					1,500	
20	146		508	966	1,474		
22	153		465	825	1,290		
33	140		460	860	1,320		
42	153		378	806	1,184		
44	160		380	810	1,190	2,340	
46	156		525	805	1,330		
49	144		562	1,000	1,562		
51	156	10.9				4,400	
53	140		535	995	1,530		
57	144	11.8				1,800	
63	156	12.8	440	935	1,375	5,400	
72	168	12.7	342	928	1,270	1,870	
77	174					3,230	
78	174	12.7					Injections ended.
80	181	13.8				8,000	
100	108	12.8	515	515	1,030		
104	93	7.4				2,400	
110	88	7.0	565	425	990		
122	88		537	253	790		
127	81	6.6	515	302	817		
132	98	7.4				2,160	
158	105	6.6	592	468	1,060		
163	110					720	Killed.

6,150 cc. of blood transfused in 59 injections; average 104 cc. per transfusion.

blood destruction, the more fragile cells from each transfusion were soon destroyed, leaving the average of those remaining higher than normal.

TABLE III.

Blood Changes in Experimental Plethora and "Plethoric Anemia."
Dog 7.

Length of time after first transfusion.	Hemoglobin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Urobilin units.	Remarks.
days	*per cent*	*millions*	*cc.*	*cc.*	*cc.*		
(Normal.)	105	6.84	550	450	1,000	400	May 17, 1920. Transfusions begun.
5	121	7.9	612	638	1,250		
9		9.1				900	
24	168	12.4	460	1,840	2,300		Splenectomized.
30	167	12.4	392	1,298	1,690		
39	146	10.7	342	948	1,290		
44	176		432	1,168	1,600		
51	176	11.6	545	1,475	2,020		
58	117	7.4	460	500	960		
65	85	5.8	560	300	860		
71	74	5.1	555	239	794		
80	98		527	448	975		
87	138	7.5	510	622	1,132		
95	195		512	1,088	1,600	4,000	
100	155		440	935	1,375		Killed.

10,450 cc. of blood transfused in 88 injections; average 118 cc. per transfusion.

TABLE IV.

Blood Changes in Fatal Experimental Plethora.
Dog 5.

Length of time after first transfusion.	Hemoglobin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Remarks.
days	*per cent*	*millions*	*cc.*	*cc.*	*cc.*	
(Normal.)	98	7.16	364	411	775	May 25, 1920. Transfusions begun.
1	102	8.12				
2	112					
4	138	9.16				
8	172	11.2				
12	200	12.8				
18	180	14.4	325	1,295	1,620	
24	172	15.2				
30	172		174	792	966(?)	
40	220	15.5	212	1,418	1,630	Death 4 days later.

5,000 cc. of blood transfused in 39 injections; average 133 cc. per transfusion.

II.

Metabolism Studies.

Although early in our work we devoted our attention solely to urobilin excretion, it later seemed advisable to study the influence of the artificial plethora and its consequent anemia on the nitrogenous metabolism, in the presence of daily parenteral introduction of large amounts of isoprotein.

The dogs used for each experiment were placed in metabolism cages, on a constant diet of raw beef heart, cracker meal, sugar, lard, bone ash, and sodium chloride, that had a known, sufficient caloric value. Water was given *ad libitum*. After 1 or 2 weeks on this special diet, if the weight of the animal remained constant, a preliminary metabolism study was made; Dogs 4 and 6 were first studied metabolically after the transfusion experiments were well in progress. The constant diet was continued between periods of metabolism study, so that although the introduced nitrogen was not actually determined, changes in elimination can reasonably be attributed to the transfused blood. Each dog was catheterized at the end of 24 hours, with the exception of one male (No. 6) chosen before the metabolic studies were contemplated. Although this renders the 24 hour periods less precise than those of the females, the length of the series studied prevents gross error in this particular experiment, if averages of several days are considered. None of the animals showed loss of weight or other ill effects during the period of metabolic study, except one (No. 5) which on account of anorexia and diarrhea could not be included in this study.

In the analysis of the urine, the total nitrogen was determined by the Kjeldahl-Gunning method, urea by Folin's aeration method, ammonia by the procedure of Van Slyke and Cullen, creatinine and creatine by Folin's method, uric acid by the Hotchkiss-Benedict modification, and total phosphates by the uranium acetate method.

Forster (18, 19) demonstrated many years ago that whole dog blood transfused into a dog in moderate amounts had a marked stability, since he failed to obtain an appreciable rise in the nitrogen output. On the other hand, foreign serum injected in the same manner is destroyed more as though given *per os*. Tschiriew (20), confirming the experiments of Forster, found that dog blood introduced by

mouth is completely metabolized in contrast to an equal amount of "living" dog blood, which may be given intravenously with little or no increase in nitrogen excretion. From these experiments, we must conclude that transfused blood protein is not immediately destroyed and that its protein is utilized when given as a food.

The nitrogenous metabolism of Dog 7 is presented (Table V) as an example of the changes that occur before and during the plethoric and anemic stages. Although the nitrogen intake in food and blood transfusions was not determined, the results of nitrogen output are

TABLE V.

Nitrogen Metabolism in Experimental Plethora.

Length of time after first transfusion.	Weight.	Urine per day.									Feces per day.
		Volume.	Specific gravity.	Total N.	NH_3 N	Urea N.	Creatinine.	Creatine.	P_2O_5	Uric acid.	N
	kg.	*cc.*		*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*
Normal (5 day period)	9.19	555	1,014	4.31	0.30	3.33	328	181	309	59	0.65
3rd and 4th days (2 day period)	9.13	635	1,013	4.28	0.26	3.34	262	233	189	54	
8th to 13th day (6 day period)	9.37	585	1,015	4.89	0.30	3.80	291	208	255	63	0.62
24th " 29th " (6 " ")	9.50	740	1,011	5.28	0.32	4.31	314	121	273	69	0.46
Splenectomized on 30th day.											
31st to 35th day (5 day period)	9.14	592	1,016	6.98	0.31	5.82	338	172	377	55	0.50
47th " 51st " (5 " ")	9.26	538	1,013	7.08	0.30	6.07	307	225	302	85	0.40
58th " 63rd " (6 " ")	9.10	478	1,018	7.31	0.31	6.28	279	143	352	72	
66th " 70th " (5 " ")	9.03	400	1,017	6.07	0.31	5.11	251	117	282	59	
73rd " 81st " (9 " ")	9.27	502	1,014	6.33	0.28	5.17	299	143	299	85	

so striking that certain conclusions can, nevertheless, be drawn. In Dog 7 during the first 10 days of the experiments, 1,200 cc. of blood were transfused, representing roughly 36 gm. of nitrogen. The nitrogen output in urine and feces for this period was practically normal, indicating that the nitrogen in excess of that accounted for by the plethoric vascular system was being stored by the body. With continued transfusions, the nitrogen output rose, but never commensurately with the amount injected. With the onset of anemia, the nitrogen excretion rose to its highest point; but during the course of the anemia, there was no such marked nitrogenous excretion.

Dog 4 was first studied metabolically while the hemoglobin was returning to normal after transfusions had been stopped. The first period, during plethora, showed a high nitrogen output; when a decrease in blood volume had occurred, the nitrogen excretion was practically normal as judged by the food intake.

In Dog 6 metabolism studies were first done while transfusions were being made, during the period of anemia. A relatively high nitrogen output was found, which decreased to about normal soon after the transfusions were stopped. During the period of recovery, there was a much lower excretion of nitrogen, which appears to be lower than normal. It is problematic at what stage the excess nitrogen of transfused blood is excreted, since there is apparently no indication from these analyses that excess, or even equal amounts of nitrogen are excreted at any time except previous to sudden development of anemia. Albumin was occasionally present in the urine during extreme plethora and at the beginning of anemia, but never in quantities that would account for the nitrogen introduced.

The creatinine, creatine, and phosphate studies give no noteworthy information about the processes concerned in blood destruction. The urea excretions paralleled the total nitrogen, but the ammonia excretion was apparently not affected. The feces nitrogen was relatively constant. It was thought that the cell destruction would be so great that a study of uric acid metabolism might be of interest, even though allantoin determinations were not made. The result, however, showed no constant or marked changes.

In the urine of all animals made extremely plethoric, bile and albumin could be detected in appreciable amounts. This was also noted during the period of anemia.

III.

Postmortem Findings.

By far the most striking change in the organs of our plethoric animals is the tremendous deposition of hemosiderin in the spleen, liver, lymph nodes, and bone marrow. Its almost complete absence from other organs is strong evidence of the important part that these organs play in the storage and elimination of the products of increased

blood destruction. Its absence from the kidney accords with the absence of hematuria or hemoglobinuria, though differing from various experimental and clinical conditions in which pigment deposition has been marked in this organ.

In animals killed or dying while transfusions were being continued, there is also, as would be expected, extreme congestion of blood vessels throughout the body. This is so marked in the viscera that details of structure often are obscured, so that it is usually impossible, for instance, to determine whether given groups of cells are packed into phagocytes or into small vessels. It is safe to say, however, that pigment phagocytosis either entirely replaces or at least is far greater than erythrocyte phagocytosis in the several organs of the hemopoietic system. The engulfing of whole erythrocytes by the phagocytes of the hemopoietic system probably only occurs in acute cases of greatly increased blood destruction and then only for a short time. In the extreme type of blood destruction that follows the administration of hemolytic serum, for instance, Pearce and Austin (21) only found erythrocytes within the phagocytes for a few days in dogs, and Karsner, Amiral, and Bock (22) found in splenectomized cats that this phase was practically complete after 48 hours. Voegtlin, Hooper, and Johnson (23) have also observed it in acute T. N. T. poisoning of dogs, but here marked fragmentation of the circulating erythrocytes and in the perfusates of spleen, liver, and bone marrow was also found.

In the spleen and lymph nodes of our animals, minute granules of hemosiderin are here and there found in large numbers free in the pulp and sinuses, and even within the blood vessels. These observations tend indirectly to support Rous and Robertson's (24) view that erythrocytes are chiefly destroyed by fragmentation into hemoglobin-containing dust, which is then removed from the circulation by the phagocytes of several organs.

The sinuses of the spleen and lymph nodes are chiefly filled by myriads of endothelial phagocytes containing hemosiderin granules of varying size. They are also numerous in the splenic pulp and even scattered through the lymphoid tissue of the lymph nodes. In smaller amount hemosiderin-bearing phagocytes persist in animals that have not been transfused for many weeks whose blood picture

has returned to normal. Hemosiderin also is found extracellularly in large clumps, apparently from fusion of pigment from phagocytes.

In the liver, hemosiderin-containing cells are also prominent; from their position between the strands of liver cells many are apparently Kupffer cells, but others occurring in Glisson's capsule and in the portal vessels are obviously the usual endothelial phagocyte. Thrombosis of bile ducts and proliferation of bile capillaries and fibrous tissue are also found. The strands of liver cells are so compressed by the congested capillaries that in many places they remain atrophied after normal conditions have been restored. A similar condition is found in some lymph nodes, where the greatly distended sinuses practically fill the node so that there is very little lymphoid tissue remaining. Grossly, lymph nodes appear enlarged, reddened or reddish brown, with a purplish center. Histologically, many of these changes are seen to be due to great congestion of the blood vessels with accumulation of the above mentioned phagocytes. In other instances, however, erythrocytes are found, sometimes in great quantities both in the central and peripheral sinuses, mingled with the usual content of endothelial, lymphoid, and plasma cells. No evidences of hemorrhage are to be found, so that the appearance of a hemolymph node is strongly suggested. In the absence of special injections, this point cannot be definitely settled, but the impression remains strong that under the need for greatly increased blood destruction, structures resembling hemolymph nodes are developed.

The bone marrow exhibits not only the same pigment deposition, but also considerable cell hyperplasia. This was not found by Itami in plethoric animals and certainly the blood picture during life points to lessened erythropoiesis. In Boycott and Douglas' animals, on the other hand, Price-Jones found a definite cellular increase, though not necessarily of cells concerned in blood formation. If this increase were only of the leucocytic series it might be correlated with the leucocytosis constantly found; but erythroblasts and unidentified cells were also increased in number. The only observations we can offer in explanation are the lack of specificity of bone marrow response, to which attention has been called elsewhere, and the impossibility of determining in a cellular bone marrow just which cells are concerned with blood cell formation and which with blood destruction. Cer-

tainly the accumulation of hemosiderin, both intra- and extracellular, and the prominence of multinuclear giant cells in the bone marrows of recovered animals point to the importance of the bone marrow as an active agent in increased blood destruction. In the recovered bone marrow, not only were multinuclear giant cells unusually numerous, but so called mature elements (polymorphonuclears and normoblasts) were considerably more prominent than in animals killed during transfusion.

In the other organs examined no noteworthy lesions were found.

In the three splenectomized dogs (Nos. 1, 3, and 7) the same changes were found in the remaining hemopoietic organs, but they were more marked. In fact, the greater pigment content of these organs, coupled with the greater urobilin excretion, suggests from another point of view Asher's (25) unconfirmed contention that the anemia of splenectomy is due to a failure to reutilize the products of destroyed erythrocytes. The relatively greater bone marrow hyperplasia in these animals has been described elsewhere (26) as occurring after splenectomy. It should be noted again, however, that this is not necessarily either wholly or in part a sign of increased hemopoiesis. In the only dog actually killed by transfusions, the signs of blood destruction were no greater, but congestion of all organs was most extreme. In fact, in the lungs, spleen, and lymph nodes, large areas of packed erythrocytes gave a picture indistinguishable from hemorrhage. It was not possible, however, to establish conclusively the immediate cause of death in this animal.

In the rabbits of this series, the outstanding feature was the widespread evidence of agglutination and thrombosis. Not only is the sudden death apparently to be explained by the extensive intravascular agglutination of blood cells and pigment, but also evidences of former thrombi are to be found within the blood vessels and in the focal necroses and fibroses of liver and spleen. The frequency of these agglutination thrombi, as well as the general frailty of the rabbit, militates strongly against its usefulness in this kind of work if experiments are to be continued over a long period. The same deposition of blood pigment in the various organs was found as in the dog, with one exception. In the rabbit, as in human hemochromatosis, the blood pigment is found in two forms: the iron-reacting granules of

hemosiderin, and minute semiopaque spicules that fail to react to the usual iron tests (probably hemofuscin). This latter pigment is especially prominent in the protoplasm of the liver cells (which in dogs were never found to contain hemosiderin), but is also found in large amounts in the various sites already mentioned.

DISCUSSION.

The relationship of artificial plethora to bone marrow activity as indicated by the percentage of reticulocytes, emphasized in the case of rabbits by Robertson (3), has been confirmed in these experiments both with dogs and rabbits. We have found that with the development of plethora the reticulocytes in the blood decreased, and that after anemia developed a reticulosis was manifest in every case. During recovery from the experimental anemia the reticulocytes were present in greatest numbers. This would indicate that while "living blood" was being supplied in excess from outside, the normal factory for erythrocytes suspended operation; the slow resumption of activity when erythrocytes were needed during the period of anemia (about 1 to 2 weeks) points either to Itami's inactivity atrophy, or to a bone marrow-inhibiting influence that required considerable time for removal.

The urobilin excretion, which under the constant conditions of these experiments can be taken as a reliable index of blood destruction, began to rise in the feces soon after transfusions were begun. It was greater during the "plethoric anemia" than in extreme plethora, but quickly lessened after transfusion was stopped. Although urobilin could not be detected in the urine during the early stages of plethora, it appeared there simultaneously with the occurrence of extreme blood destruction. This accords with the findings of other investigators that fecal urobilin may be greatly increased before it appears in the urine.

Details of the manner in which blood is normally destroyed in the body are still open to question. It was hoped that the addition of large quantities of blood and the subsequent destruction of this excess blood would disclose the underlying mechanism of blood destruction. Phagocytosis has been considered as a possible means of blood destruction, and the importance of endothelial cells in the spleen and the

Kupffer cells of the liver has been pointed out by Pearce and Austin (21). Rous and Robertson (24) found an appreciable phagocytosis in dogs, but believe this would not suffice for normal blood destruction. It is unquestionable, however, that greatly increased phagocytosis of blood pigment occurs in artificial plethora, as it does in many forms of increased blood destruction. The fragmentation of erythrocytes, which they consider an important factor in the destruction of blood, was not observed by us in the circulation in amounts greater than that seen under normal conditions. It is, of course, possible that as we did not enter into the special procedures that they undertook, this may have played a greater part than we appreciate. The relation of the postmortem findings to this topic has already been discussed.

It is well known that parenteral introduction of certain foreign substances will cause the formation of antitoxins, agglutinins, and hemolysins, and that antibodies may sometimes be formed after injection of protein from the same species. It is quite obvious that the first of these factors, namely antitoxins, may be eliminated in these experiments. As previously mentioned, Robertson (3) and Kambe and Komiya (4) have shown the presence of antibodies capable of destroying erythrocytes, even of the same species, in large numbers during plethora in rabbits; and isohemolysins and isoagglutinins have been produced in cats after compatible transfusions (5).

In a further attempt to explain the mechanism of this "plethoric anemia" by the production of agglutinins or hemolysins, ten rabbits were transfused by us with 5 to 20 cc. of compatible rabbit blood daily, according to the method previously described. Their blood changes corresponded in the main with those we have reported in dogs, but at no time were we able to demonstrate the presence of isoagglutinins or isohemolysins. Thinking that in our previous study on dogs, antibodies might have developed, but been absorbed by the daily repeated injections, we discontinued transfusions on the anemic rabbits for varying lengths of time before the antibody tests were made. Carefully controlled quantitative tests, with the complement fixation method, still continued negative. We are therefore unable to aid in the solution of this phase of the problem, and have no explanation as to why, like Boycott and Douglas and other observers, we were unable to demon-

strate either agglutinins or hemolysins while blood was obviously being destroyed in greatly increased quantities and in spite of the fact that in the rabbits agglutinative thrombi were found at autopsy. Faulty technique, the most obvious explanation, we endeavored to guard against most carefully. The ability of transfused rabbits to survive was extremely slight for reasons already discussed under the section on pathological anatomy.

In regard to the effect of splenectomy on plethora, and on the consequent anemia, one cannot but be impressed by the more constant and striking anemia of the dogs splenectomized during the course of the transfusions (Nos. 6 and 7) than of those that were not splenectomized (Nos. 4 and 5). In view of Hektoen's demonstration (27, 28) that under certain conditions antibody formation is lowered after splenectomy, and of our inability to demonstrate antibodies at any time, this anemia of splenectomized dogs can hardly be based on such grounds; but we feel confident that some relationship exists between the loss of the spleen and the marked anemia that developed. It is also of interest that "plethoric anemia" is harder to produce in dogs than in rabbits, animals whose spleens are relatively much smaller (0.05 per cent of body weight as against 0.25 per cent of dogs).

The two chief points of interest in the blood volume studies are: (1) the confirmation of previous findings as to the constancy of the plasma volume under widely varying conditions, and (2) the remarkable adaptability of the organism in accommodating itself to rapid increases in total blood volume reaching as high as 250 per cent of the normal.

The metabolism studies have pointed out a marked retention of nitrogen by the organism for a long period of time. We are not able to learn from these experiments how and when this excess nitrogen is eliminated from the body. This retention and the failure of the hemoglobin to rise appreciably during the first 10 days of transfusions with a corresponding lack of increased nitrogen elimination would seem to indicate the existence of a mechanism for the storage of considerable excess blood and its decomposition products.

The possibility of finding a true plethora followed by anemia in clinical medicine is illustrated by a recent report by Freund (29) of a case studied in Krehl's clinic.

After incurring syphilis and other infectious diseases, the patient developed a typical Vaquez polycythemia of 135 per cent hemoglobin, and blood count of 9,200,000 red cells. Several months later the blood picture gradually changed to that of pernicious anemia, with megaloblasts, stippled red cells, normoblasts, and urobilinuria. This condition was next followed by exhaustion of the hemopoietic system with absence of nucleated red cells, myelocytes, and platelets, a steadily falling hemoglobin, and finally death from aplastic anemia.

SUMMARY AND CONCLUSIONS.

1. The effects of repeated transfusions of blood on the blood-destroying and blood-forming apparatus of normal and splenectomized dogs and rabbits have been described. An anemia which developed despite continued blood transfusions in two dogs splenectomized during plethora has also been studied.

2. The decrease or absence of reticulocytes from the blood stream during plethora and their increase during "plethoric anemia" are evidently due to depression and activation of bone marrow activity. The response of the bone marrow is not immediate upon the onset of anemia, but is delayed for several days.

3. The blood volume studies have served to emphasize the constancy of plasma volume under extreme experimental conditions, and the adaptability of the circulatory system to large increases in total blood volume.

4. Blood destruction and elimination, as measured by urobilin excretion, are greatly increased during the stage of plethora, but still more so during "plethoric anemia."

5. Despite intravenous introductions of large quantities of nitrogen in the form of whole blood, the total nitrogen, urea, and ammonia in the urine and feces are not raised appreciably for some time after the onset of plethora. The normal organism is apparently able to store large quantities of blood or its decomposition products. Upon the onset of a "plethoric anemia," there is an increase in urinary total nitrogen and urea excretion, which was lowered during the course of the anemia. Albuminuria is also found at this time. Other nitrogenous constituents and phosphates show no striking changes.

6. Blood pigment, chiefly in the form of hemosiderin, is deposited in enormous quantities in the spleen, liver, lymph nodes, and bone marrow. It occurs chiefly in phagocytes, though in late stages large

extracellular masses are found. Increased pigment deposition can still be found several months after transfusions have been stopped.

7. Phagocytes containing erythrocytes are found rarely, if at all, and only in the acute cases, but their occurrence may be greatly masked by the coexistent congestion.

8. In splenectomized dogs the tendency to "plethoric anemia" is much more apparent, although a direct connection between the two events is not established. In rabbits, whose spleens constitute only 0.05 per cent of body weight, "plethoric anemia" is more easily produced.

9. In splenectomized animals pigment-bearing phagocytes are especially prominent in the liver, although lymph nodes and bone marrow apparently share in the extra work caused by the absence of the spleen. Lymph nodes with some of the characteristics of hemolymph nodes were found in various localities in all animals that had been made plethoric.

10. In rabbits blood pigment is deposited in the hemopoietic organs in large amounts, but under the conditions of our experiments, the picture and the experiment have been constantly complicated by early fatal intravascular agglutination and thrombosis. In the rabbit, as in human hemochromatosis, the pigment is found in two forms: hemosiderin granules, and smaller, dark spicules that do not react to the usual iron stains (probably hemofuscin). The latter pigment is also found seeded through the cells of the liver parenchyma.

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